

BEHAVIORAL RECOVERY FROM ESSENTIAL AMINO ACID DEFICIENCY

By

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Abstract of Dissertation Presented to the Graduate School
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For decades it has been known that rats can adjust their nutritive intake based on need. While this phenomenon has been extensively described for sodium deficiency, other micronutrient deficiencies have not been thoroughly explored. Essential amino acid (EAA) deficiency represents a relevant model to describe adaptive changes in behavior resulting from deficiency. The purpose of these experiments was to characterize lysine (LYS) deficiency using a variety of behavioral paradigms. In *Experiments 1A* and *1B*, licking during 10-s trials was measured in lysine-deficient (LYS-DEF), threonine-deficient (THR-DEF), and control (CON) rats before and after 23-h intake tests. The intake tests provided an opportunity for the deficient rats to associate the taste of the EAA with its repleting postingestive consequences. EAA-deficient rats did not show increased

licking to the deficient EAA in any of the brief-access tests, but they did initiate significantly more trials than CONs. The LYS-DEF rats also had elevated intake of the deficient EAA in long-duration tests. In *Experiments 2A, 2B, and 2C*, two-bottle EAA preference was determined while licking was measured with 6-s resolution. Meal pattern analyses revealed that in both one- and two-EAA choice tests, LYS-DEF rats consumed significantly more LYS than CONs by initiating more bouts; bout size and within-bout drinking rate did not differ between groups. Cumulative licking analyses on *Day 1* of stimulus presentation revealed that LYS-DEF rats consumed more LYS relative to CONs by 30 min when LYS and water were presented, and by 90 min when LYS and THR were presented; at no time did the groups differ when THR and water were presented. In *Experiments 3A and 3B*, rats were tested using a progressive ratio (PR) reinforcement schedule to examine the "reward value" (represented by PR break point) of NaCl and LYS, in sodium-deficient and LYS-DEF rats, respectively. In both cases, deficient rats had higher break points relative to nondeficient CONs. Experience with LYS did not increase break point for LYS-DEF rats. Collectively, these findings suggest that LYS deficiency results in an appetite that develops over time, is at least somewhat chemospecific, and does not enhance palatability of LYS.

CHAPTER 1 GENERAL INTRODUCTION

In his book, "The Wisdom of the Body" (1932), Walter Cannon described the impressive ability of organisms to maintain internal constancy. He provided examples of animals regulating physiological variables (e.g., fluid levels, temperature, and maintenance of mineral, glucose, fat, and protein levels) through both innate and learned mechanisms. With Cannon's book, the concept of homeostatic regulation became the leading perspective in feeding and nutrition research. Although relatively little concrete evidence supports the exact idea of strict homeostatic control over feeding behavior (particularly in cases of deficiency), popular culture widely accepts the notion that when the body is lacking in a particular nutrient, an individual will develop an adaptive craving for a foodstuff containing that nutrient.

Early clinical studies support the belief in so called "body wisdom." For example, Davis (1929) allowed recently weaned infants ($n=3$) to choose the quantity and type of food ingested and then related these measures to the condition of

the infant after six months. This report provides fascinating case studies indicating that good health in infants can be achieved when they are allowed to control what is ingested. Although the choice of foods was limited to only "healthy", safe commodities (meats, seafood, cereals, eggs, milk, fruits, vegetables, and salt), restricting the generality of the experiment, this publication has been used to promote the idea that animals and humans (even as young as 6 months of age) will eat complete diets, grow normally, and overcome deficiency when allowed to make individual food choices.

Richter's work has also been used as evidence for the occurrence of adaptive food choices. In addition to maintaining nutritive status under normal conditions, Richter reported examples of humans sustaining health in the face of unique physiological challenges. Specifically, Wilkins and Richter (1940) presented a compelling case of a child with adrenal gland damage who maintained electrolyte balance by consuming huge quantities of salt. Sadly, when salt intake was restricted by allowing access to only a normal hospital diet, the child developed signs of deficiency and died. In another case report, they described an adult patient with Addison's disease who had an enormous

salt appetite, manifested by adding incredible amounts of salt to foods.

Anecdotal evidence has also been used to support the concept of body wisdom. Pregnant women are often cited for their cravings and have been the topic of survey. For example, Hook (1978) found that of 250 women asked to list their food cravings, 18.4% listed ice cream and 4.0% listed pickles. As with most cravings, these are believed to be well-founded and can easily be justified as adaptive based on the fact that the requirements for calories (fat in ice cream) and salt (in pickles) are increased during pregnancy. While these and similar examples are intriguing, more rigorous experimental tests must be conducted to support or refute the existence and generality of body wisdom, and to potentially uncover the underlying mechanisms.

The work that comes closest as support for the apparent ease with which animals can regulate their own nutrition was conducted by Richter (1942). He studied the effects of feeding "cafeteria" diets to rats. Animals were allowed to choose from several cups containing solid food sources and between 8 and 11 solutions for 500 days. Compared with rats raised on standard laboratory chow, the cafeteria fed rats maintained equal growth rates, reproduced, were active, and showed no outward signs of deficiency.

The specific nutrient deficiency paradigm is an experimental model germane to the idea of feeding-related body wisdom. Specifically, animals can be experimentally depleted of a selected essential nutrient (i.e., micronutrient) and the variables involved in guiding behavioral recovery from the deficiency can then be carefully examined. This *micronutrient* model can be contrasted with *macronutrient* manipulations. Frequently, overall deprivation and/or depletion of selected *macronutrients* (i.e., protein, carbohydrate, or fat) are used to examine the regulation of feeding. While the macronutrient model has provided a great deal of information concerning hunger and satiety mechanisms, the micronutrient model, from a sensory standpoint, may potentially challenge the organism further, demanding a high degree of specificity, both behaviorally and physiologically. Furthermore, micronutrient manipulations may represent a situation more analogous to that which exists in nature where specific nutrients occur (or are lacking) in mixtures. Thus, focusing on micronutrient deficiency may in turn provide insight into the myriad of factors that control food selection. While more natural scenarios are desirable, animal models are typically used to study specific nutrient

deficiency because maximal control over the diet can be obtained and deficiencies can be readily induced.

In the present experiments, rats were exposed to specific essential amino acid (EAA) deficiencies in order to 1) demonstrate further enhanced ingestive responsiveness based on dietary need, 2) determine under what testing conditions this enhanced responsiveness occurs, 3) reveal the time course of the increased ingestion, 4) define meal pattern adjustments that may accompany the elevated intake, and 5) describe the reinforcement efficacy of a deficient stimulus using an operant behavioral task. It was hoped that addressing these specific questions would yield general conclusions concerning how animals use behavioral means to recover from other nutrient deficiencies.

CHAPTER 2 GENERAL METHODS AND MATERIALS

Subjects

Adult male Sprague-Dawley rats (Charles River, Wilmington, MA) were used in all experiments. Rats were singly housed in hanging, wire-mesh cages in a colony room where temperature was automatically maintained. All experimental manipulations were performed during the light phase of the 12-h light-dark cycle (lights on 0600-1800). Unless otherwise specified, rats had ad libitum access to Purina Rat Chow 5001 (Ralston-Purina, St. Louis, MO) and tap water.

Apparatus

Gustometer

The gustometer (fully described by Spector, Andrews-Labenski, & Letterio, 1990) was a specially designed computer-automated taste testing apparatus that allowed delivery of controlled volumes (~5 μ l per lick) of fluid and

precisely measured the number of licks elicited by a given stimulus. Up to 12 liquid stimuli can be placed in separate reservoirs and delivered, in turn, through solenoid valves to a spout located behind a 1-cm slit in one wall of the chamber. Upon tongue contact with the spout, a low current (< 50 nA) electrical circuit was completed and a lick was recorded. At the termination of a stimulus trial (trial length is determined by experimenter), the spout rotated out of the rats' reach, was rinsed with distilled water, cleared by pressurized air, and rotated back into place for the animal to initiate another trial. This cleaning procedure required ~6 s.

The gustometers that were used in *Experiments 3A* and *3B* were modified to include two identical levers (BRS/LVE, Laurel, MD) located 80 mm on either side of the stimulus-access slit, 40 mm above the floor of the chamber. A cue light covered with translucent glass is positioned above each lever.

Twenty-Three-Hour Intake Pattern Measures

The apparatus used to measure licking during 23-h periods is a revised version of that described previously by Spector and Smith (1984). Briefly, 16 Hoeltge hanging wire mesh rat cages were modified to include two slits in the back of the cage allowing access to two stainless steel

drinking spouts located just outside the cage. Thus, the rat's tongue must protrude just beyond the slit opening to contact the spout. The rat was required to extend its head inside a feeding compartment on the front of the cage for access to food. Phototransistors and infrared light-emitting diodes (LED) were positioned so that licks on either of the two spouts and entries into the feeder interrupted a beam. The beam interruptions were transmitted to a computer and recorded in 6-s bins. Intake patterns were measured for 23 h, allowing a 1-h period each day for refilling solution bottles and food cups.

Diet-Induced Deficiency

Essential amino acid deficiency was accomplished by limiting the level of the amino acid in the diet. Body weight was measured daily and used as an index of deficiency. Rats in a given experiment first had ad libitum access to a basal diet for seven days. Both basal diets consisted of ~11% protein and contained all the EAAs, but were slightly limiting in either lysine (LYS; *Experiments 1A, 2A, 2B, 2C, 3A, 3B*) or threonine (THR; *Experiments 1B*). The purpose of pre-feeding the basal diet was to reduce endogenous stores of circulating free amino acids and proteins so that deficiency could be induced rapidly upon

feeding the EAA-deficient diet (see Gietzen & Beverly, 1992).

Following the seven day pre-feeding period of basal diet, rats were divided into two groups, matched for body weight and basal diet food intake. The control (CON) group was fed a complete, amino acid-balanced diet consisting of ~20% protein. The other group was given an EAA-deficient diet matched for caloric and nitrogen levels.

In experiments that included LYS deficiency, the CON diet contained 20.4% protein, consisting of a total of 2% LYS. The EAA-deficient group was fed a lysine-deficient (LYS-DEF) diet that consisted of 19.4% protein, containing a total of only 0.1% LYS. Nitrogen levels were equated between the two diets by adding the nonessential amino acid glycine (GLY) to the LYS-DEF diet.

In *Experiment 1B*, the CON diet contained 20.8% protein, consisting of a total of 0.6% THR. The EAA-deficient group was fed a threonine-deficient (THR-DEF) diet that consisted of 19.4% protein (a total of 0.2% THR). The two diets were made equal in nitrogen content by adding the nonessential amino acid proline to the THR-DEF diet. The ingredients for all 6 diets used in the studies are presented in Table 2-1. All experimental diets were obtained from Harlan Teklad (Madison, WI) in powdered form and kept refrigerated.

Table 2-1. Diet Formulas

INGREDIENT (g/kg diet)	LYS basal	LYS-CON	LYS-DEF	THR basal	THR-CON	THR-DEF
L-arginine HCl	10.0	10.0	10.0	10.0	10.0	10.0
L-asparagine	10.0	10.0	10.0	10.0	10.0	10.0
L-serine	3.5	3.5	3.5	3.5	3.5	3.5
L-proline	10.0	10.0	10.0	10.0	10.0	13.74
glycine	10.0	10.0	16.4	10.0	10.0	10.0
L-glutamic acid	30.0	30.0	30.0	30.0	30.0	30.0
L-alanine	3.5	3.5	3.5	3.5	3.5	3.5
L-histidine HCl H ₂ O	3.0	11.0	11.0	3.0	11.0	11.0
L-isoleucine	4.0	14.0	14.0	4.0	14.0	14.0
L-leucine	4.0	18.0	18.0	6.0	20.0	20.0
L-lysine HCl	4.0	20.0	1.0	8.0	24.0	24.0
L-methionine	3.0	9.0	9.0	3.0	9.0	9.0
L-cystine	2.0	6.0	6.0	2.0	6.0	6.0
L-phenylalanine	4.5	14.5	14.5	4.5	14.5	14.5
L-tyrosine	2.5	8.5	8.5	2.5	8.5	8.5
L-threonine	4.0	8.0	8.0	2.0	6.0	2.0
L-tryptophan	1.0	3.0	3.0	1.0	3.0	3.0
L-valine	5.0	15.0	15.0	5.0	15.0	15.0
sucrose	256.4	223.0	230.03	254.2	220.73	220.82
corn starch	512.79	445.89	460.06	508.29	441.46	441.63
corn oil	50.0	50.0	50.0	50.0	50.0	50.0
mineral mix, AIN-76	50.0	50.0	50.0	50.0	50.0	50.0
sodium acetate	6.8	17.1	8.5	8.5	18.8	18.8
vitamin mix, Teklad	10.0	10.0	10.0	10.0	10.0	10.0
ethoxyquin	0.01	0.01	0.01	0.01	0.01	0.01
choline chloride	-	-	-	1.0	1.0	1.0

Chemical Stimuli

The taste stimuli for all of the experiments were prepared daily using room temperature distilled water. The amino acid stimuli (e.g., LYS, THR, GLY) were obtained from Sigma Chemical Company (St. Louis, MO). Stimulus concentrations were selected based on Pritchard and Scott (1982) and were within the dynamic behavioral range described by long-term, two-bottle preference-aversion functions. That is, a preferred (or neutral in the case of LYS) and an avoided concentration were chosen for the present experiments. Furthermore, electrophysiological recordings (Pritchard & Scott, 1982) confirmed that the concentrations used were above the neural thresholds for these stimuli.

Biological grade sucrose, used during training in *Experiments 1A* and *1B*, and sodium chloride (NaCl), used in *Experiment 3B*, were obtained from Fisher Scientific (Fair Lawn, NJ).

CHAPTER 3
EXPERIMENT 1: SHORT-DURATION VERSUS LONG-DURATION INTAKE

Introduction

In some instances, specific nutrient deficiency produces a very robust appetite for the deficient or limiting substance (see Denton, 1984; Rozin, 1965). The classic specific appetite, first described experimentally by Richter (1936), occurs as a consequence of sodium deficiency; animals deficient in sodium invariably search for and ingest sodium salts. Denton (1984) provides several accounts of animals in the wild seeking out salt as a result of this deficiency. In addition to naturalistic observations, the appetite for sodium, arising from inadequate levels of this mineral in the body, has been characterized in numerous controlled experiments using rat subjects (for reviews see Denton 1984; Schulkin, 1991). In general, three defining features of sodium deficiency have emerged from the literature.

First, sodium deficiency appears to produce a behavioral response that is innate or unconditioned. In

other words, no obvious learning is required before a sodium-deficient animal will seek out and ingest sodium. Evidence for this feature comes from the strikingly short latency to drink sodium salts that is displayed by sodium-depleted subjects (see Handal, 1965; Nachman, 1962). Assuming that the rat subjects have never been depleted of sodium before the experimental manipulation, coupled with the negligible latency of enhanced responsiveness to this cation, Wolf (1969) argued that the behavior of sodium-deficient rats must be innate. While the existence of an inborn behavior is difficult to prove, it is likely that the rats had no experience prior to testing that would allow them to learn that salt was the substance that would produce repletion. The speed at which sodium-depleted rats respond to sodium-containing solutions supports the notion that this behavior is innate.

Second, sodium appetite is very specific for salts containing this cation. When sodium-deficient rats are presented with both sodium and nonsodium salts, they respond preferentially to the former. This is true in both short-duration tests (i.e., brief-access; see Breslin, Spector, & Grill, 1993, 1995; Handel, 1965; Kriekhaus, 1970; Kriekhaus & Wolf, 1968; Markison, St. John, & Spector,

1995; Nachman, 1962) and long-duration testing paradigms (i.e., 24-h intake; Fregly, 1958; Richter & Eckert, 1938).

The third defining feature of sodium appetite is that it appears to be taste-guided. The importance of gustatory signals in sodium appetite is strongly supported by results generated from brief-access tests that minimize the influence of postingestive signals. When rats have only limited access to NaCl and immediate responsiveness to the stimulus is measured, several investigators have shown that the appetite is impaired following transection of the chorda tympani branch of cranial nerve VII (Breslin, Spector, & Grill, 1993, 1995; Markison, St. John, & Spector, 1995). The peripheral gustatory system must be intact for the sodium-deficient rat to respond appropriately to sodium in short-duration tests.

The behavioral characteristics of other specific nutrient deficiencies have not been examined as systematically as sodium deficiency. Therefore, in the present experiments we chose to examine EAA deficiency utilizing behavioral paradigms similar to those that have been applied to the study of sodium deficiency. Specifically, behavioral responsiveness during LYS (*Experiment 1A*) and THR (*Experiment 1B*) deficiency was examined in two parallel experiments. First, immediate

licking responses to amino acid stimuli and water presented in short-duration trials (a paradigm used by Breslin, Spector, & Grill, 1993, 1995; Markison, St. John, & Spector, 1995) were measured. If EAA deficiency produced behavioral outcomes in any way similar to sodium deficiency, one would expect rats to show elevated licking to the deficient EAA relative to both the other stimuli in the array and to the nondepleted controls. Such an outcome would support the existence of an innate, specific EAA appetite. Furthermore, these studies examined whether or not increased responsiveness to the needed amino acid would occur in long term, 23-h intake tests conducted over a series of days.

Method

Subjects

In *Experiment 1A*, 17 rats weighing an average of 374 g (± 13 g) at the start of the experiment were used. In *Experiment 1B*, 18 rats weighing an average of 203 g (± 12 g) were used. Rats had ad libitum access to tap water and pelleted Purina Chow (5001) prior to the start of the experiments. When the experiments began, rats were given distilled water and powdered Purina Chow (5001).

General Procedure

The procedures for *Experiments 1A* and *1B* are summarized in Table 3-1. The rats were habituated to the laboratory environment for at least nine days. Water bottles from the home cage were then removed. The following day, pelleted food was replaced with powdered chow (Purina 5001) and gustometer training began. For six days, the rats were trained to lick in the gustometer (see *Gustometer Training* below). During the first four days of training the rats were water-deprived. On *Day 4*, after the training session, home cage water bottles were replaced; training on *Days 5-6* was conducted without water deprivation. The day after gustometer training was completed, rats were given the basal diet for seven days. Rats were then divided into two groups, matched as closely as possible for total number of trials taken in the gustometer during the last two days of training, the average number of licks taken to water and sucrose during those days, body weight, and chow intake on the last day of basal diet feeding. One group was then fed the CON diet and the other group was fed the EAA-deficient diet for the next 10 days. Following dietary depletion, the rats were tested in the gustometer for their licking responses to an array of taste stimuli including LYS, THR, GLY, and distilled water. Two days after *Gustometer Test 1*,

two-bottle preference for the limiting EAA (i.e., either LYS or THR) versus distilled water was measured for 23-h periods. Preference for the limiting amino acid was measured for a total of six days in *Experiment 1A* (LYS deficiency) and five days in *Experiment 1B* (THR deficiency). Solution intake was measured for only five days in *Experiment 1B* due to limited amounts of the diet; we were unable to obtain more from our commercial source in sufficient time to extend testing. The second day after bottles were removed from the cage, EAA-deficient rats and their CONs were tested again in the gustometer exactly as before (*Gustometer Test 2*). *Gustometer Test 2* concluded the THR deficiency experiment. In the LYS deficiency experiment however, rats were fed their respective diets for 10 more days prior to a third gustometer test (*Gustometer Test 3*). Two days later, 23-h, three-bottle intake was measured for six days. Body weight and food intake were measured daily throughout both experiments. Liquid intake was only measured during 23-h two- and three-bottle testing.

Table 3-1. Procedures for Experiments 1A and 1B

	EXP 1A: LYS-DEF	EXP 1B: THR-DEF	PURPOSE
N	LYS-DEF: n=9 CON: n=9	THR-DEF: n=9 CON: n=9	
Initial Body Weight	387 g $\pm$ 16.5	223 g $\pm$ 16.3	
Gustometer Training	5 days- water & sucrose	5 days- water & sucrose	Train rats to lick in gustometer.
Dietary Manipulation	7 days-basal diet 10 days-exp diets	7 days-basal diet 10 days-exp diets	Deplete rats of a specific EAA.
Gustometer Test 1	10-s licking to water, lys, gly, thr	10-s licking to water, lys, gly, thr	Innate, specific, taste-guided EAA appetite?
Two-choice Preference	6 days- lys vs. water	5 days- thr vs. water	EAA appetite expressed in long-term test?
Gustometer Test 2	(same as Gust test 1) 10-s licking to water, lys, gly, thr	(same as Gust test 1) 10-s licking to water, lys, gly, thr	Is an EAA appetite observed in a short-term paradigm after long-term experience with the EAA and repletion?
Dietary Manipulation	10 days- deficient diet		Ensure that rats are EAA-DEF during gustometer testing.
Gustometer Test 3	(same as Gust test 1 & 2) 10-s licking to water, lys, gly, thr		Is an EAA appetite observed in a short-term paradigm when rats are depleted during testing?
3-choice Preference	lys, thr, water		Will LYS-DEF rats prefer lysine in the presence of another taste?

Gustometer Training

Following ~23 h of water deprivation, rats were placed in the gustometer for 30 min where they had continuous access to distilled water from the drinking spout. This gustometer habituation phase was implemented for two days (*Days 1 and 2*). On the third and fourth days of gustometer training, the rats were left in the gustometer for 40 min during which the drinking spout rotated in and out of the animals' reach according to a timed trial structure. When the rat licked the spout once, a 10 s trial was initiated. During these sessions, the rat had access to water and 0.3 M sucrose presented in random order. This preferred concentration of sucrose was included in the stimulus array during gustometer training to provoke further sampling in conditions where the animals would not be deprived of water prior to the session. After training on *Day 4*, water bottles were replaced on the home cage. On *Days 5 and 6*, rats were again placed in the gustometer for 40 min sessions where they had 10 s trials of distilled water and 0.3 M sucrose. The only difference between *Days 3-4* and *5-6* was that during the latter training days the animals were not water-deprived. During the final two days, spout training was conducted without water deprivation because this more accurately simulated the subsequent test conditions.

Gustometer Tests 1, 2, & 3

The number of licks to an array of taste solutions, presented in 10 s trials was measured during one 40 min session for each rat. Rats were not water-deprived during testing. The stimulus array included distilled water and two concentrations each of LYS (0.2 and 1.0 M), GLY (0.1 and 1.0 M), and THR (0.1 and 0.7 M). As during training, one lick on the drinking spout initiated a 10 s trial. In *Experiment 1A* (LYS deficiency), rats were always presented with 0.2 M LYS for the first trial. In *Experiment 1B* (THR deficiency), the first trial for every rat was always 0.1 M THR. Following the first trial, the stimuli were presented in randomized blocks of seven. The limiting amino acid was presented first in an attempt to motivate the EAA-deficient rats to further sample the tastants from the spout. In *Experiment 1A*, three identical gustometer tests were conducted on different days. In *Experiment 1B*, two identical gustometer tests were conducted on different days.

Twenty-Four-Hour Two-Bottle Intake Testing

Rats were presented with two graduated cylinders on the home cage. One bottle contained distilled water and the other bottle contained the limiting amino acid (0.2 M LYS in *Experiment 1A* and 0.1 M THR in *Experiment 1B*). Intake was

measured to the nearest 0.5 ml every 23 h for six days in *Experiment 1A* and for five days in *Experiment 1B*. Solutions were mixed fresh and replaced daily at approximately 1000 h. Bottle positions were alternated daily.

Twenty-Four-Hour Three-Bottle Intake Testing

The last measurement in *Experiment 1A* was a series of six, three-bottle 23-h intake tests. LYS-DEF rats and their nondepleted counterparts were presented with three graduated cylinders on the home cage. One bottle contained distilled water, the other bottle contained 0.2 M LYS, and the third bottle contained 0.1 M THR. For six days, intake to the nearest 0.5 ml was measured 23 h after the bottles were put on the cage the previous day. Solutions were mixed each day and replaced at approximately 1000. Bottle position was rotated daily in a counterbalanced design.

Data Analysis

Body weights were converted to percent of initial body weight by dividing each animals weight on a given day by the animal's weight on the day before the basal diet was presented. These values were then compared using two group by day analyses of variance (ANOVAs) for two phases of the experiment (the seven days of basal diet feeding and the first 10 days of experimental diet feeding). Subsequent

independent Student's t-tests were performed between groups for each day of the experiment.

The number of trials initiated during the gustometer tests was analyzed using two-way (group by test session) ANOVAs. Main effects were further investigated using Student's t-tests.

For the gustometer tests, the number of licks in response to a given stimulus was averaged over the session for each rat. Rats that took fewer than two trials of each of the seven stimuli presented during a gustometer test were excluded from the lick data analysis; less than two trials per stimulus would represent an unreliable sample of behavior. This was the case with 4 of the 8 CONS in *Experiment 1A* and 6 of the 9 CONS in *Experiment 1B*. Thus the average number of licks to the array of test stimuli were compared between the different gustometer test within the same group using two-way (test session by stimulus) ANOVAs. Independent Student's t-tests were conducted to determine differences in licking between tests for each stimuli.

For the 24-h two-bottle intake tests, intake for the EAA was divided by total intake (water + EAA) and multiplied by 100 to obtain the percentage of intake that was the EAA. This was done for each day that intake was measured. The

EAA preference scores were then analyzed by two-way (group by day) ANOVAs. Significant group by day interactions were investigated using independent Student's t-tests to compare preference scores between the groups on each of the test days.

For the 24-h three-bottle intake tests (*Experiment 1A* only), intake for LYS was divided by total intake (water + LYS + THR) and multiplied by 100 to obtain the percent of intake that was LYS. This was done for each of the six days intake was measured. The LYS preference scores were then analyzed using a two-way (group by day) ANOVA. Significant main effects of day were investigated using independent Student's t-tests collapsed over group.

Results

Body Weight

Figure 3-1 depicts the mean body weights ($\pm$ se) of the EAA-deficient rats and their CON groups for both *Experiments 1A* and *1B*. As expected, ANOVAs (group by day) over the seven days of basal diet feeding indicated that there were no differences between the dietary groups in either experiment. There were significant main effects of day in both experiments indicating that all groups of rats gained

weight over the seven days of basal diet feeding (*Experiment 1A*: $F(6,90)=152.0$, $P<0.001$; *Experiment 1B*: $F(6,96)=38.83$, $P<0.001$). Clearly, the basal diet was sufficient to support growth.

ANOVAs (group by day) comparing body weight after the rats had been divided into separate dietary groups and fed either the CON diet or the EAA-deficient showed significant main effects of group and day, and significant interactions for both experiments (see Table 3-2 for ANOVA results). Subsequent Bonferroni-adjusted t-tests revealed that the rats fed the LYS-DEF diet had significantly lower body weights relative to their CON group by the second day after the diet was presented (all $P<0.05$). Similarly, Bonferroni-adjusted t-tests indicated that the rats fed the THR-DEF diet had significantly lower body weights relative to their CON group by the first day after the diet was presented (all $P<0.001$). In both experiments the rats fed the EAA-deficient diet had significantly lower body weights for all remaining days in the experiments (all $P<0.001$). These body weight results demonstrate the effectiveness of these diets to induce deficiency.

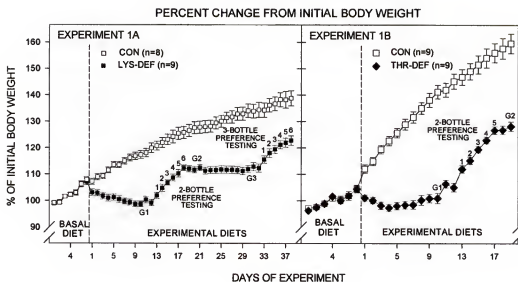


Figure 3-1. Experiments 1A and 1B: Body Weight

The mean ($\pm$ SE) percent of initial body weight plotted as a function of day. Control rats in both experiments (CON; open squares) had steady growth rates. Lysine-deficient rats (LYS-DEF; closed squares in left panel) only gained weight when they were given lysine solution on the home cage (during two- and three-bottle preference testing). Threonine-deficient rats (THR-DEF; closed diamonds, right panel) only grew when they were given threonine solution on the home cage (during two-bottle intake testing). Note that both groups of EAA-deficient rats showed slight but nonsignificant increases in body weight following each of the gustameter tests (G1, G2, G3). Rats in *Experiment 1A* underwent additional testing resulting in more days in the experiment. The scales on the horizontal axes are different because *Experiment 1A* was conducted over a period of 45 days while *Experiment 1B* continued for only 26 days.

Table 3-2. Two-way ANOVA results for body weight.

Exp. diet feeding	Experiment 1A	Experiment 1B
group	F(1,15)=57.51, P<0.01	F(1,16)=163.8, P<0.01
day	F(9,135)=15.42, P<0.01	F(9,144)=112.0, P<0.01
interaction	F(9,135)=94.76, P<0.01	F(9,144)=99.42, P<0.01

Gustometer Tests

Figure 3-2 shows the number of trials that the rats initiated in all gustometer tests. Clearly, EAA-deficient rats were more motivated (represented by trial number) in this task compared to the nondepleted CONs. In *Experiment 1A*, a group by test session ANOVA revealed a significant main effect of group ($F(1,15)=9.06$, $P<0.01$) indicating that the LYS-DEF rats took significantly more trials than the CON rats during all three gustometer tests. In *Experiment 1B*, a group by test session ANOVA revealed a significant main effect of group ($F(1,16)=19.85$, $P<0.001$) and a significant main effect of test session, $F(1,16)=31.82$, $P<0.001$). Although, the THR-DEF rats took more trials in both gustometer tests relative to the CONs, a paired t-test (collapsed over group) revealed that the rats took more trials during *Gustometer Test 1* than *Gustometer Test 2* ($T(17)=5.62$, $P<0.001$).

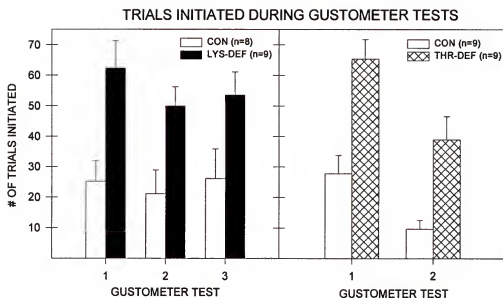


Figure 3-2. Experiments 1A and 1B: Trials initiated during Gustometer Testing

The mean ($\pm$ SE) number of trials initiated for the dietary groups plotted for each gustometer test. Control rats in both experiments (CON; open bars) initiated fewer trials than both the lysine-deficient rats (LYS-DEF; closed bars in left panel) in *Experiment 1A* and the threonine-deficient rats (THR-DEF; cross-hatched bars, right panel) in *Experiment 1B*.

Because CON animals did not initiate many trials during gustometer testing, comparisons between sessions were limited to the EAA-deficient groups. One LYS-DEF, 4 LYS CON, 2 THR-DEF, and 6 THR CON rats were excluded from the gustometer licking analysis because of a failure to meet the trial criterion.

Figure 3-3 depicts lick data for the LYS-DEF rats (*Experiment 1A*) taken from all three gustometer tests. A test session by stimulus ANOVA revealed a significant main effect of session ($F(2,14)=4.87$, $P<0.05$) and of stimulus ($F(6,42)=22.44$, $P<0.001$). One-way ANOVAs conducted for each stimulus revealed a significant difference among sessions only at 0.7 M THR, $F(2,14)=9.14$, $P<0.01$). Paired t-tests indicated that the LYS-DEF rats licked less to 0.7 M THR during *Test 1* and *Test 2* relative to *Test 3*. There were no other significant differences among test sessions.

Figure 3-4 depicts lick data for the THR-DEF rats (*Experiment 1B*) taken from both gustometer tests. A session by stimulus ANOVA revealed significant main effects of session ($F(1,6)=42.61$, $P<0.001$) and stimulus ($F(6,36)=32.28$, $P<0.001$). Paired t-tests done at each stimulus revealed significant differences between the two sessions at 0.2 M LYS, 1.0 M LYS, and 0.7 M THR (all $P<0.01$); licking was significantly less during *Test 2*.

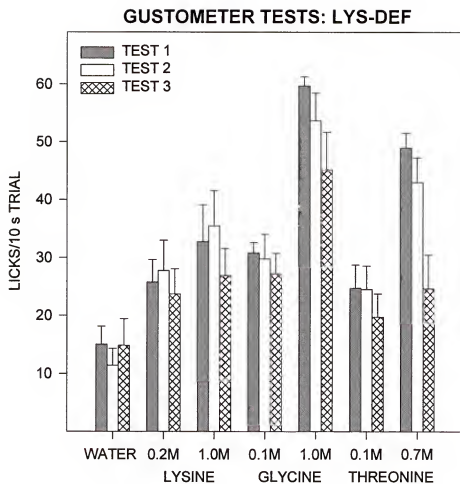


Figure 3-3. Experiment 1A: Gustometer Tests

The mean ($\pm$ SE) number of licks lysine-deficient (LYS-DEF) rats took in response to each stimulus during each of three gustometer tests.

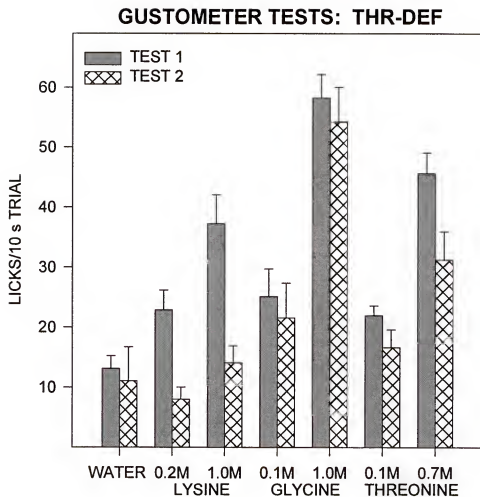


Figure 3-4. Experiment 1B: Gustometer Tests

The mean ($\pm$ SE) number of licks threonine-deficient (THR-DEF) rats took in response to each stimulus during each of two gustometer tests.

In general, the number of licks that EAA-deficient rats took to each of the stimuli presented during gustometer testing remained relatively stable across the test sessions. These findings indicate that the manipulations interspersed between tests (two- and three-bottle preference testing) had very little effect on the immediate lick responsiveness elicited by these tastants.

In addition to the comparisons made between test sessions in each experiment, the two EAA-deficient groups were compared between the experiments (see Figure 3-5). A group by stimulus ANOVA revealed no significant main effect of group or interaction. Lick responsiveness did, however, vary among stimuli ($F(6,78)=46.71$, $P<0.001$). Bonferroni-adjusted paired t-tests revealed that rats licked significantly more to every stimulus relative to water (all $P<0.001$).

Twenty-Four-Hour Two-Bottle Intake Tests

Figure 3-6 displays intake (panels 1 & 2)and LYS preference (panel 3) resulting from the two-bottle tests in *Experiment 1A*. Separate group by day ANOVAs revealed that LYS-DEF rats drank more LYS ($F(1,15)=8.05$, $P<0.05$) and less water ($F(1,15)=10.57$, $P<0.001$) relative to CONs, resulting in greater LYS preference ($F(1,15)=13.13$, $P<0.01$). There

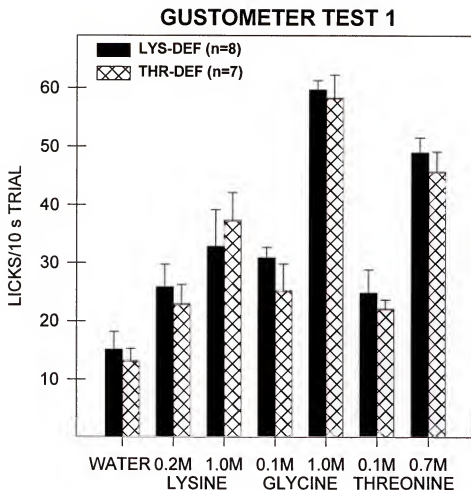


Figure 3-5. Experiments 1A and 1B: Gustometer Test 1

Mean ($\pm$ SE) number of licks for lysine-deficient (LYS-DEF; closed bars) and threonine-deficient (THR-DEF; cross-hatched bars) rats in response to each stimulus during the initial gustometer test. These data are re-plotted from Figures 3-3 and 3-4 for purposes of comparison.

TWO-BOTTLE INTAKE

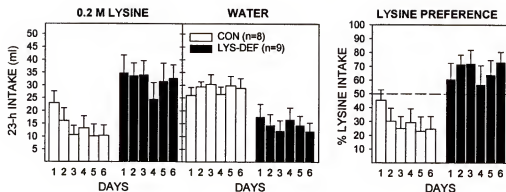


Figure 3-6. Experiment 1A: Two-Bottle Intake and Preference

Mean (+ SE) 23-h intake in milliliters of 0.2 M lysine (left panel) and water (middle panel) and the mean percent of total intake that was lysine (+ SE; right panel) plotted by days of testing for control (CON; open bars) and lysine-deficient (LYS-DEF; closed bars) rats.

were no significant main effects of test day or any interactions.

Figure 3-7 represents amount consumed of each stimulus and THR preference taken from *Experiment 1B*. The THR-DEF group ingested more THR ($F(1,15)=19.15$, $P<0.01$) and less water ($F(1,15)=14.57$, $P<0.01$) compared to CONs. Furthermore, the deficient rats preferred THR more than CONs ($F(1,15)=16.24$, $P<0.01$). For THR intake there was also a significant main effect of day ($F(4,60)=5.43$, $P<0.001$). Paired t-tests, collapsed over group, revealed that rats drank more THR on Day 1 relative to Days 3, 4, and 5 (all $P<0.05$). There were no significant interactions.

Twenty-Four-Hour Three-Bottle Intake Tests

Figure 3-8 depicts intake of 0.2 M LYS, 0.1 M THR, and distilled water over the six days of 3-bottle preference testing. Individual group by day ANOVAs showed significant main effects of group for LYS intake ($F(1,15)=18.82$, $P<0.01$), water intake ($F(1,15)=9.52$, $P<0.01$), and LYS preference ($F(1,15)=12.29$, $P<0.01$), indicating that the LYS-DEF rats drank more total LYS and less water than CONs, producing higher LYS preference scores. The groups did not differ in responsiveness to THR. There were, however, main effects of day for LYS ($F(5,75)=11.60$, $P<0.01$) and LYS

TWO-BOTTLE INTAKE

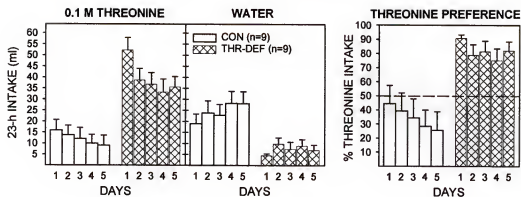


Figure 3-7. Experiment 1B: Two-Bottle Intake and Preference

Mean (+ SE) 23-h intake in milliliters of 0.1 M threonine (left panel) and water (middle panel) and the mean percent of total intake that was threonine (+ SE; right panel) plotted by days of testing for control (CON; open bars) and threonine-deficient (THR-DEF; cross-hatched bars) rats.

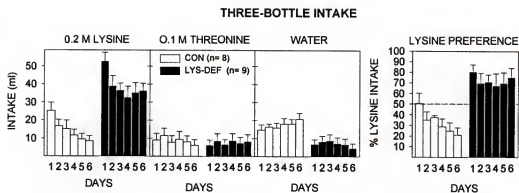


Figure 3-8. Experiment 1A: Three-Bottle Intake and Preference

Mean (+ SE) 23-h intake in milliliters of 0.2 M lysine (first panel), 0.1 M threonine (second panel) and water (third panel) and the mean percent of total intake that was lysine (+ SE; forth panel) plotted by days of testing for control (CON; open bars) and lysine-deficient (LYS-DEF; cross-hatched bars) rats.

preference ($F(5,75)=4.10$, $P<0.01$). Paired t-tests collapsed over group showed that LYS intake was significantly decreased on Days 2, 3, 4, 5, and 6 relative to Day 1 and preference was significantly lower on Days 3 and 5 compared to Day 1. There were no significant interactions in any of the ANOVA results.

Discussion

These experiments suggest that LYS and THR deficiency do not produce behavioral responses analogous to those of sodium deficiency. Indeed, acute sodium deficiency produces an appetite with unique properties that do not generalize to other specific nutrient deficiencies, or at least not to EAA deficiency. The initial short-duration licking test was conducted to determine if EAA-deficient rats would express an innate (i.e., unconditioned) and specific increase in licking to a needed commodity as has been observed in sodium-deficient rats (Breslin, Spector, & Grill, 1993 and 1995; Markison, St. John, & Spector, 1995). In contrast to sodium deficient rats consuming sodium, the pattern of licking to the array of amino acids was not altered as a function of deficiency; LYS-DEF rats did not specifically increase responsiveness to LYS, nor did THR-DEF rats show elevated licking to only THR. In fact, the entire profile

of responsiveness across the stimulus array was nearly identical between groups deficient in either LYS or THR (see Figure 3-8). Unfortunately, we were unable to confidently compare the responses during brief-access trials in EAA deficient animals to those in nondepleted CONs because the nondeficient, non-water-deprived CON rats did not initiate a sufficient number of trials to serve as a meaningful comparison. This is apparent from the analysis of the number of trials initiated by the groups in the gustometer tests (see Figure 3-2). In the absence of water deprivation most CON rats did not sample the drinking spout. Tests that do not rely on appetitive behaviors for stimulus delivery and only measure consummatory responding (e.g., intra oral infusion) may circumvent this sampling problem.

There were, nonetheless, significant difference in the number of trials initiated in nondeficient versus EAA-deficient animals. This suggests that, although deficient rats do not specifically ingest the nutrient missing in their diet in the short-duration test, they can still be characterized as "motivated." EAA deficiency seems to increase appetitive behavioral responding in a general sense.

Although deficiency did not influence immediate lick responsiveness, LYS- and THR-deficient rats expressed a

preference for the amino acid that was absent from their diet (see Figures 3-5, 3-6, and 3-7). Differences in long-duration tests occurred presumably because consumption is under the control of both oral-sensory and postingestive cues. These preference results support and extend the work of others who have shown that rats can compensate for deficient diets by ingesting the missing nutrient when it is offered to them in solution. This has been shown with vitamin B deficiency (Richter, Holt, & Barelare, 1937), protein deficiency (Halstead & Gallagher, 1962), histidine deficiency (Rogers and Harper, 1970), tryptophan deficiency (Mori, Kwada, Ono, & Torii, 1991) and LYS deficiency (Torii, Mimura, & Yugari, 1986; Mori, Kwada, Ono, & Torii, 1991).

The findings from the later gustometer tests were somewhat surprising. After the two-bottle intake tests, we measured short-duration licking responses in the gustometer again. The rationale here was that the two-bottle tests would allow the deficient rats to learn an association between the sensory cues (i.e., the taste) of the limiting EAA and repletion. We hypothesized that this learning would be manifested in a specific increase in licking to the deficient amino acid in the short-duration test. Even after this opportunity to associate the taste and positive postingestive consequences of the missing EAA, however, rats

still did not increase licking to the EAA. One explanation is that *Gustometer Test 2* was only 2 days after the intake testing and it is possible that the rats were LYS replete. If this were the case, physiological state (i.e., repleted) could account for the lack of effect during the second test in *Experiments 1A* and *1B*. However, the results from the third gustometer test (*Experiment 1A*), conducted after 10 more days of deficient diet feeding, revealed that rats still did not show enhanced responsiveness to LYS.

There are at least two possible explanations for the failure to see a conditioned preference in the gustometer tests. First, it could be argued that at the concentrations presented, these amino acids are not detectable by the taste system and therefore rats are unable to distinguish them from water in short-duration tests. This explanation appears unlikely based on both behavioral and electrophysiological findings. In short-duration tests, which are postulated to reflect oral-sensory control, several investigations have shown differential responsiveness to amino acids as a function of concentration. Additionally, when licking during short-duration trials was measured in the gustometer tests, rats took significantly more licks to each and every amino acid stimulus relative to water. In the case of GLY, Grill,

Flynn, and Schwartz (1987) showed that nondeprived rats increased ingestive taste reactivity as a function of concentration (0.03 M, 0.1 M, 1.0 M). Electrophysiological measures demonstrate that the gustatory system responds to the concentrations of amino acids used in these experiments. Pritchard and Scott (1982) found chorda tympani whole-nerve thresholds (the weakest concentration that evoked a response in the whole nerve) to be 0.8 mM for LYS, 20 mM for THR, and 3.5 mM for GLY. Clearly, the concentrations employed in the present experiment fall within the range that stimulates the chorda tympani nerve. This was reflected in lick responsiveness to LYS that was significantly above that of water.

A second possibility for the absence of an effect of deficiency on short-duration licking even after experience with repletion, relates to changes (or lack thereof) in "palatability" with EAA deficiency. Palatability has been an elusive term in psychology and specifically in the study of ingestive behavior. While palatability cannot be directly measured, several indices have been proposed. For example, it has been suggested that the rate of licking to a stimulus (especially during the early portions of an ingestive episode) may reflect its palatability. Rats tend to have higher rates of licking to more preferred stimuli.

Alternatively, the palatability of a stimulus may emerge in terms of reflexive oromotor facial responding in taste reactivity tests (Grill & Norgren, 1978). In this case, more preferred stimuli elicit greater levels of consummatory responding and acceptance (e.g., tongue protrusions, mouth movements).

With a mathematical model for the control of ingestion, Davis and Levine (1977) described how an analysis of lick rate can be used to distinguish between the relative palatability versus the postingestive effects of various stimuli. For example, Davis (1989) showed that although overall intake of two sucrose concentrations did not differ, initial lick rate was much higher to 0.8 M sucrose versus 0.05 M sucrose.

In addition to the inherent features of the stimulus, physiological state has been shown to affect measures of palatability. Breslin, Kaplan, Spector, Zambito, and Grill (1993) showed that lick rate to a range of NaCl concentrations increased when rats were tested following sodium deficiency. Taste reactivity has also been used to assess alterations in palatability in response to changes in physiological state. Berridge, Flynn, Schulkin, and Grill (1984) found that intraorally infused 0.5 M NaCl (a normally aversive concentration) elicited significantly more

ingestive responses and significantly fewer aversive responses following sodium depletion relative to before depletion.

In the context of the present experiments it is conceivable that depleting rats of EAAs may change lick rate to the needed commodity. This would be analogous to sodium deficient rats showing higher lick rates to sodium. However, in the LYS-DEF rat, lick rate during 10 s trials was not altered as a function of deficiency and may reflect the lack of a shift in palatability with deficiency.

In sum, both LYS-deficient and THR-deficient rats show elevated intake of the limiting amino acid as measured during long-duration (i.e., 23-h) intake tests. Additionally, this intake may be specific, at least in the case of LYS deficiency since LYS-DEF rats preferred LYS even when it was presented with LYS, THR, and water. It is unlikely that EAA deficiency promotes an "innate" appetite; LYS-DEF rats do not increase intake of the deficient EAA immediately in short-duration tests. Furthermore, even after experience with the limiting amino acid rats do not show elevated lick rates in short duration-tests. The question remains then, what mechanisms are responsible for the adaptive elevation in intake that occurs as a result of EAA deficiency?

As suggested above, 24-h intake tests are limited in the information they provide. Specifically, long-duration tests provide a measure of the outcome of feeding behavior and do not reveal details regarding the actual behavior leading to the amount ingested. Although, these data confirmed that an appetite exists, further investigation is necessary to determine the mechanisms guiding the behavior.

CHAPTER 4
EXPERIMENT 2: MEAL PATTERN CHANGES WITH LYSINE DEFICIENCY

Introduction

Rats fed a diet deficient in an EAA preferred the missing amino acid when it was presented in solution on the home cage for 23-h periods. Furthermore, recovery from the deficiency was paralleled by a gain in body weight that corresponded in time to the elevated intake. Although deficiency influenced behavior over long measurement intervals (i.e., hours), there were no observable differences between dietary groups in lick responsiveness during short-duration tests.

These findings reveal that EAA deficiency produces behavioral responses different from those of sodium appetite and suggests two hypotheses that merit further examination. First, the appetite for the missing EAA is not immediate and therefore recognition of the needed stimulus is not likely an innate process. Instead, the enhanced LYS consumption as a result of EAA deficiency appears to develop over time (i.e., is observable after 23 h) and may be learned.

Second, because EAA-deficient rats did not alter licking responses (in brief access tests) to the limiting EAA even after experience with its taste and postingestive consequences, it is possible that deficiency did not enhance the palatability of the stimulus. Based on these findings and interpretations, I thought it would be fruitful to characterize explicitly how EAA appetite is expressed in long-duration tests. Such a detailed characterization of the appetite should offer insight into the appropriateness of the hypotheses suggested above.

Although LYS-DEF rats increased intake of LYS in 23-h intake tests, this provides little information about *how* they increased their intake. Therefore, in order to understand the moment-by-moment behavioral changes that accompany repletion from an EAA deficiency, a fine-grained analysis of 23-h intake (meal pattern analysis) was employed. Potentially, meal pattern analysis can point to the behavioral mechanisms that support LYS intake. The amount consumed over the course of a day is a result of the combination of bout size and bout number. One advantage of analyzing licking during ingestion is that it allows for the determination of how various gustatory, physiological, pharmacological, or surgical manipulations influence these parameters of intake (Davis, 1989). It is possible that the

enhanced intake in the LYS-DEF rat will be the result of either increases in bout size (perhaps reflecting a shift in the palatability of lysine) or as a result of an increase in bout number. Additionally, rate of ingestion during a bout can be quantified and has been shown by others to reflect sensory properties of the test stimulus. For example, Spector and Smith (1984) found that in two-bottle tests, rate of drinking during a bout increased monotonically with sucrose concentration. An analysis of the meal patterns of LYS-DEF rats ingesting LYS would lend information about the hedonic evaluation of this stimulus and provide useful information about exactly how rats adjust intake of LYS in the face of this augmented nutritional need.

Another advantage of lick pattern analysis is that the development of an appetite can be followed. At what point in time do LYS-DEF rats begin drinking more LYS relative to their nondeficient counterparts? It has been shown that when sodium solutions are presented to sodium-deficient and nondeficient rats, behavioral differences can be seen as early as 5 s into the session (Nachman, 1962; Handal, 1965). Given that LYS-DEF rats should not show an amplified response to LYS immediately, the characterization of the appetite's time course is important. Such information could

offer insight into the time required for the LYS-DEF rat to associate the taste of LYS with signals of repletion.

Additionally, in the following experiments the chemospecificity of elevated intake in LYS-DEF rats was examined. This was done to explore the nature of the appetite stimulated by specific EAA deficiency. In particular, does amino acid deficiency promote an appetite that is distinct for the needed amino acid or does it generalize to other amino acids? This type of "general appetite" has been shown to occur in calcium-deficient rats. Calcium deficiency induced up to an eightfold elevation in daily intake of 0.3 M NaCl relative to rats fed a control diet (Tordoff, Ulrich, & Schulkin, 1990).

In the following experiments, each of these questions were addressed: (1) What adjustments in meal pattern (e.g., bout number, bout size), if any, accompany the deficiency-induced enhanced LYS responsiveness? (2) Does enhanced intake occur in response to another nonlimiting amino acid, THR? (3) What is the developmental time course of the enhanced LYS responsiveness (determined by cumulative licking over 23 hours) during both one- and two-amino acid choice tests? Potentially, the results generated from the LYS-DEF rat may be generalizable to behavioral recovery from other specific nutrient deficiencies.

Method

Subjects

In *Experiment 2A*, 14 rats weighing an average of 364 g (± 22.6) at the start of the experiment were used. Sixteen rats, weighing 415 g (± 20.4) were used in *Experiment 2B*. Sixteen rats were also used in *Experiment 2C*; they weighed 283 g (± 8.0) at the start of the experiment. Rats had ad libitum access to powered Purina chow (5001) and distilled water prior to the beginning of the experiments and during the spout-licking habituation phase. When the experiments began, rats were given the experimental diets and distilled water; During testing, taste solutions were also presented on the cage (described below).

General Procedure

The procedures for *Experiments 2A*, *2B*, and *2C* are briefly summarized below in Table 4-1. The rats in all three experiments were habituated to the Hoeltge cages (modified for intake pattern measures) for at least 3 days prior to presentation of the basal diet. The length of the sipper tubes was varied over the three days such that on *Day 1*, the tubes were long enough to protrude through the slit

and into the cage. On *Day 2*, the tubes were slightly shorter and on *Day 3*, the animals were presented with the tube length that was used throughout the rest of the experiment. These tubes did not protrude into the cage; the rat was required to extend its tongue through the slit, outside of the cage, to make contact with the spout. This was done to prevent accidental beam breaks. During the spout-licking habituation and the dietary manipulation phases of the experiments, rats had access to two bottles filled with distilled water. Following habituation, all rats were fed the basal diet for seven days. For the next 10 days, the animals in the top two rows of cages were fed the LYS-DEF diet, while the bottom two rows of rats were fed the CON diet. Body weight was measured each day and used to monitor the deficiency. This was done because other investigators have shown that when rats are fed a diet that is devoid or low in a single EAA, food intake is decreased and weight loss occurs (e.g., Elvehjem & Krehl, 1955; Harper, 1958).

After the depletion phase of the experiment, LYS-DEF and CON rats were presented with a choice of solutions. In *Experiment 2A and 2B*, rats were presented with distilled water and one amino acid solution, 0.2 M LYS or 0.1 M THR, respectively. In *Experiment 2C*, rats were presented with a

choice between two amino acids, 0.2 M LYS and 0.1 M THR. Body weight, diet intake, and solution intake were measured daily at ~1400 h. During approximately a 1-h period, fresh solutions were made and bottle sides were alternated.

Table 4-1. Procedures for Experiments 2A, 2B, and 2C.

	EXP 2A: LYS vs WAT	EXP 2B: THR vs WAT	EXP 2C: LYS vs THR
N	LYS-DEF: n=7 CON: n=7	LYS-DEF: n=8 CON: n=8	LYS-DEF: n=8 CON: n=8
Initial Body Weight	364 g $\pm$ 22.6	415 g $\pm$ 20.4	283 g $\pm$ 8.0
Spout- Licking Habituation	Sipper tube lengths varied over 3 days.	Sipper tube lengths varied over 3 days.	Sipper tube lengths varied over 3 days.
Dietary Manipulation	7 days-basal diet 10 days-exp diets	7 days-basal diet 10 days-exp diets	7 days-basal diet 10 days-exp diets
Choice Test 4 days	0.2 M lysine distilled water	0.1 M threonine distilled water	0.2 M lysine 0.1 M threonine
Choice Test 4 days		0.2 M lysine distilled water	

Data Analysis

Body weights were converted to percent of initial body weight by dividing each animal's body weight on a given day by that animal's body weight on the day before the basal diet was presented. These values were then compared using two group by day ANOVAs for the two diet presentation phases of the experiment (seven days of basal diet feeding and 10 days of experimental diet feeding). Subsequent independent

(unpaired) Student's t-tests were performed between groups for each day of the experiment.

Intake measured in milliliters and LYS preference (LYS intake divided by total intake, then multiplied by 100) were examined with group by day ANOVAs. Significant day main effects were examined using Bonferroni-adjusted paired t-tests (collapsed over groups). Significant group by day interactions were examined using Bonferroni-adjusted unpaired t-tests comparing groups on each of the four test days.

In addition to intake measured in milliliters, the number of licks (in 6 s bins) that each rat had in each port (i.e. left bottle, right bottle) during 23-h periods was determined. For the cumulative licking analysis, these data were summarized into 15 min intervals, providing the number of licks taken to each of the stimuli during 92 consecutive 15 min bins. Unpaired t-tests at each of the 92 time intervals were used to compare cumulative licking on Day 1 between dietary groups.

For meal pattern analysis, the lick data were summarized into bouts. Typically rats eat and drink in bouts of behavior, which can be operationally defined. A bout was defined using the following three criteria: 1) a bout started with 3 licks within a 6-s bin, 2) a bout ended

when there were no licks for 5 consecutive minutes, and 3) bouts less than 30 licks were excluded from the bout analysis. These criteria allow for a characterization of intake, describe how the animals consumes the test stimuli, and typically account for greater than 95% of the total number of licks. In other words, these criteria truly capture the feeding behavior and accurately describe it. When the total lick data were divided into meal patterns, three variables were examined: bout number, bout size (licks/bout), and ingestion rate (licks/s). Increases in overall intake can occur as a function of increases exclusively in either bout number or bout size (licks per bout), or a combination of changes in both variables.

Meal pattern parameters were examined with two-way (group by day) ANOVAs. Significant day main effects were examined using Bonferroni-adjusted paired t-tests (collapsed over groups). Significant group by day interactions were examined using Bonferroni-adjusted unpaired t-tests comparing groups on each of the four test days.

Although, as already shown, LYS-DEF rats increase their daily LYS intake on average, there is some variability among individuals. A few rats show only modest increases or do not increase intake at all. It would not be meaningful to include such rats in the analysis of the meal patterns

because the goal of this analysis is to describe changes in meal pattern that *correspond* to the adaptive preference for LYS. Therefore, rats were excluded from the cumulative licking and meal pattern analyses if they did not fall above the 95% confidence interval of the CON group with respect to both four day LYS intake and four day LYS preference. One rat in *Experiment 2A*, one rat in the LYS versus water test in *Experiment 2B*, and two rats in *Experiment 2C* were eliminated from the cumulative licking and meal pattern analyses.

Results

Body Weight

Figure 4-1 shows the percent of initial body weight plotted for each day in the experiment for both dietary groups. The top panel shows the results from *Experiment 2A* in which rats were presented with LYS and water, the middle panel shows the results from *Experiment 2B* in which rats were presented with THR and water, and the bottom panel shows the results from *Experiment 2C* in which rats were presented with LYS and THR.

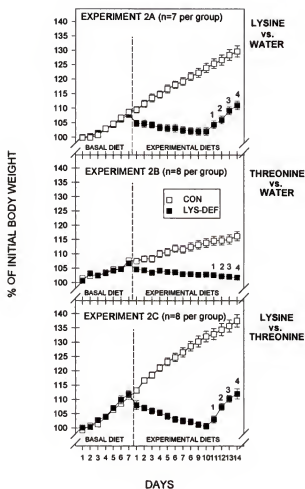


Figure 4-1. Experiments 2A, 2B, and 2C: Body Weight

The mean ($\pm$ SE) percent of initial body weight plotted as a function of day. Control rats in all three experiments (CON; open squares) had steadily increasing growth rates. Lysine-deficient rats (LYS-DEF; closed squares) in all three experiments showed growth rates comparable to CONs during basal diet feeding, but lost weight when they were presented the deficient diet. In *Experiments 2A* (top panel) and *2C* (bottom panel), lysine-deficient rats grew when they were given lysine solution on the home cage indicated by the numbers 1-4 above the days corresponding with testing. In *Experiment 2C* (middle panel), 1-4 indicates the test days that rats were presented with threonine and water.

The body weight results from all three experiments were similar. There were no differences between the body weights of the dietary groups during the seven-day basal diet phase in any of the three experiments. The basal diet contains sufficient protein and is appropriately balanced with regard to amino acid content as demonstrated by the fact that all rats gained weight over the seven days of basal diet feeding (*Experiment 2A*: $F(1,6)=132.0$, $P<0.001$; *Experiment 2B*: $F(1,6)=113.0$, $P<0.001$; *Experiment 2C*: $F(1,6)=205.0$, $P<0.001$). Groups did differ as a function of what diet they were fed during the first 10 days when they were switched to the experimental diets (*Experiment 2A*: $F(1,12)=61.97$, $P<0.001$; *Experiment 2B*: $F(1,14)=29.57$, $P<0.001$; *Experiment 2C*: $F(1,14)=129.5$, $P<0.001$). Subsequent Bonferroni-adjusted t-tests revealed that the rats fed the LYS-DEF diet had significantly lower body weights relative to their CON group by the first day after the diet was presented in *Experiment 2C* (all $P<0.05$) and by the second day in *Experiments 2A* and *2B* (all $P<0.05$). Consistent with findings from *Experiment 1A*, these results also indicate that feeding rats a diet with a reduced level of LYS produces a substantial decline in body weight indicative of the deficiency.

Experiment 2A: Lysine versus Water

Figure 4-2 shows that, overall, LYS-DEF rats consumed significantly more LYS than did CONS ($F(1,12)=16.39$, $P<0.01$) and less water ($F(1,12)=19.98$, $P<0.001$). This pattern of two-bottle intake, which emulates that seen in *Experiment 1A*, resulted in a significantly greater LYS preference ($F(1,12)=34.26$, $P<0.001$). There were also significant main effects of day for both water intake ($F(3,36)=3.09$, $P<0.05$) and LYS preference ($F(3,36)=4.86$, $P<0.01$). Bonferroni-adjusted paired t-tests, collapsed over groups, indicated that LYS preference was significantly lower on *Day 3* relative to *Day 1*.

Meal pattern analysis revealed that LYS-DEF rats increased LYS intake by initiating significantly more LYS bouts relative to CONS ($F(1,11)=25.32$, $P<0.001$). Furthermore, LYS-DEF rats took significantly fewer water bouts ($F(1,11)=17.82$, $P<0.001$), thus resulting in a greater percentage of total bouts as LYS ($F(1,11)=56.24$, $P<0.001$).

Figure 4-3 displays bout number and intake enabling a direct comparison. It should be noted that intake has been replotted excluding the rat in the LYS-DEF group that did not express a clear LYS preference according to the criteria detailed in the *data analysis* section. This pattern of

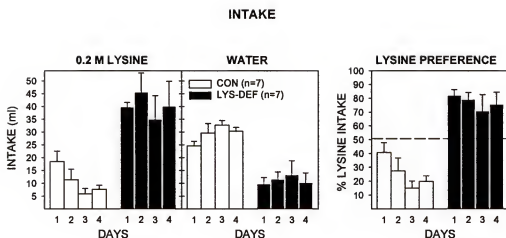


Figure 4-2. Experiment 2A: Two-Bottle Intake and Preference

Mean (+ SE) 23-h intake in milliliters of 0.2 M lysine (left panel) and water (middle panel) and the mean percent of total intake that was lysine (+ SE; right panel) all plotted by days of testing for control (CON; open bars) and lysine-deficient (LYS-DEF; closed bars) rats.

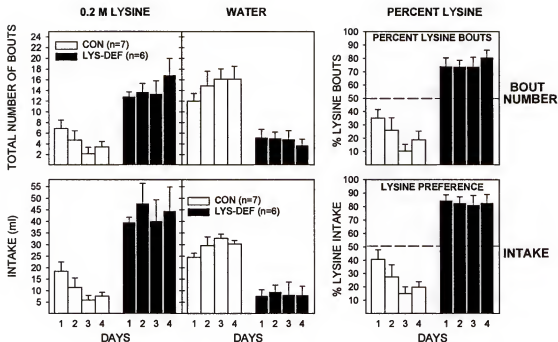


Figure 4-3. Experiment 2A: Bout number and Intake

The top panel shows mean (+ SE) number of bouts for 0.2 M lysine (left panel) and water (middle panel) and the mean percentage of the total bouts that were lysine (+ SE; right panel) all plotted by days of testing for control (CON; open bars) and lysine-deficient (LYS-DEF; closed bars) rats. For purposes of comparison the bottom panel depicts mean (+ SE) 23-h intake in milliliters of 0.2 M lysine (left panel) and water (middle panel) and the mean percent of total intake that was lysine (+ SE; right panel) all plotted by days of testing for control (CON; open bars) and lysine-deficient (LYS-DEF; closed bars) rats. Rats that were excluded from the bout analysis were also excluded from these data.

results convincingly reveals that bout number paralleled the changes observed for intake.

The other measures from the meal pattern analysis (i.e., bout size and ingestion rate) did not appear to be substantially influenced by deficiency. Figure 4-4 represents bout size in terms of the average number of licks taken per bout. Bout size to LYS did not vary between groups. For water, however, CONs took significantly larger bouts compared to LYS-DEF rats ($F(1,10)=6.16$, $P<0.05$). Figure 4-5 reveals that drinking rate was unaffected by LYS deficiency for both stimuli. Although, there was a significant main effect of day for LYS ($F(3,24)=4.63$, $P<0.01$). Post hoc tests (Bonferroni-adjusted paired t -tests, collapsed over groups) revealed that LYS drinking rate was significantly slower on Day 1 relative to Days 3 and 4 (all $P<0.05$).

Figure 4-6 shows the number of cumulative licks that rats took during Day 1 of LYS and water presentation. LYS-DEF rats took significantly more licks to LYS relative to CONs by 30 min after sampling the LYS (all $P<0.05$) and CONs took significantly more cumulative licks to water at minute 405 (6 h, 45 min) and each interval thereafter.

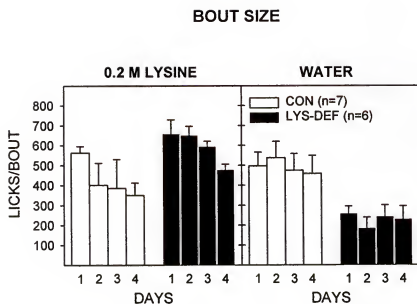


Figure 4-4. Experiment 2A: Bout size

Mean (+ SE) bout size in terms of licks per bout for 0.2 M lysine (left panel) and water (middle panel) plotted by the four days of testing for control (CON; open bars) and lysine-deficient (LYS-DEF; closed bars) rats.

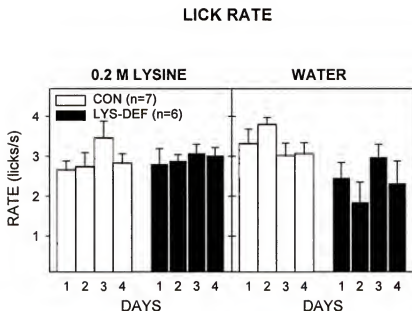


Figure 4-5. Experiment 2A: Lick rate

Mean (+ SE) lick rate represented as licks per second for 0.2 M lysine (left panel) and water (middle panel) plotted by the four days of testing for control (CON; open bars) and lysine-deficient (LYS-DEF; closed bars) rats.

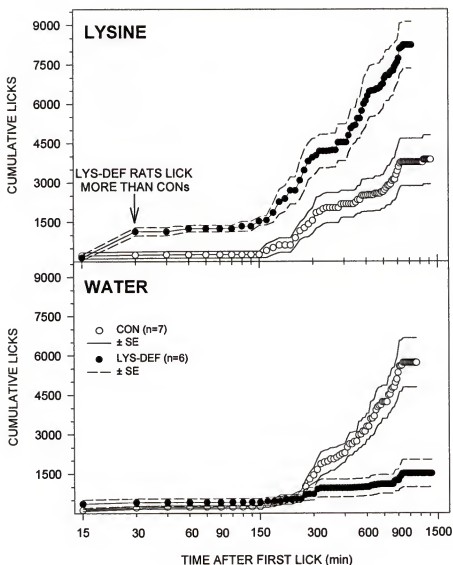


Figure 4-6. Experiment 2A: Cumulative licks on Day 1 of lysine presentation

Mean number of cumulative licks for lysine (top panel) and water (bottom panel) plotted in 15 minute intervals during the first 23-h amino acid presentation period. Time is plotted on a logarithmic scale to more clearly resolve the onset of the appetite. Controls (CON; open circles) take fewer cumulative licks to lysine and more to water relative to lysine-deficient (LYS-DEF; closed circles) rats. Solid lines represent SEs for controls. Dashed lines represent SEs for the lysine-deficient group.

Experiment 2B: Threonine versus Water

Not surprisingly LYS-DEF rats did not increase intake of THR, a nonlimiting amino acid, in the current context (see Figure 4-7). There were no significant main effects of group for THR intake, water intake, or THR preference, nor were there any significant main effects of day or interactions. Bout number and bout size also did not differ between groups.

Although there were no group differences for intake, LYS-DEF rats, at some time interval, might have preferred THR. This possibility, however, was not supported by the cumulative lick analysis. Figure 4-8 shows the number of cumulative licks that rats took during Day 1 of THR and water presentation. At no time interval did the dietary groups differ in responsiveness to THR or water.

Experiment 2B: Lysine versus Water

As expected, there were no differences between the dietary groups in response to THR. Thus, LYS intake was measured to ensure that the typical increase in LYS responsiveness would be observed. As demonstrated by Figure 4-9, LYS-DEF rats drank more LYS ($F(1,14)=30.11$, $P<0.001$) and less water ($F(1,14)=28.72$, $P<0.001$) relative to CONS,

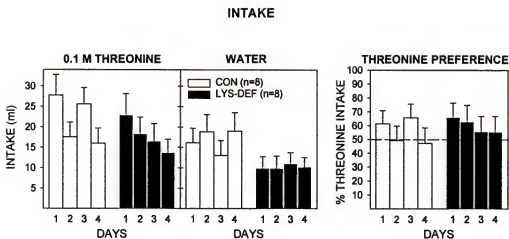


Figure 4-7. Experiment 2B: Two-Bottle Intake and Preference

Mean (+ SE) 23-h intake in milliliters of 0.1 M threonine (left panel) and water (middle panel) and the mean percent of total intake that was threonine (+ SE; right panel) plotted by the four days of testing for control (CON; open bars) and lysine-deficient (LYS-DEF; closed bars) rats.

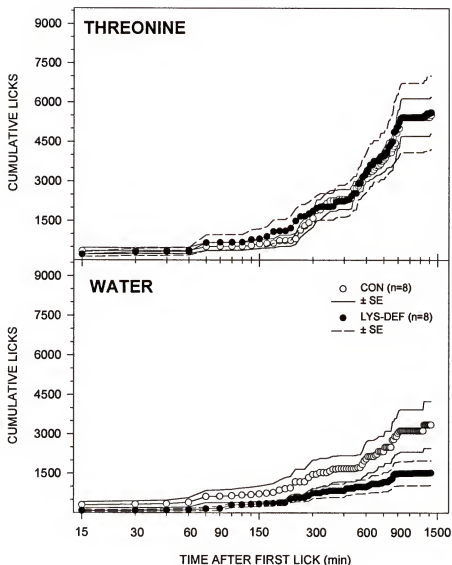


Figure 4-8. Experiment 2B: Cumulative licks on Day 1 of threonine presentation

Mean number of cumulative licks for threonine (top panel) and water (bottom panel) plotted in 15 minute intervals during the first 23-h amino acid presentation period. Time is plotted on a logarithmic scale to more clearly resolve the onset of the appetite. Controls (CON; open circles) and lysine-deficient (LYS-DEF; closed circles) rats do not differ in responsiveness to threonine. Solid lines represent SEs for controls. Dashed lines represent SEs for the lysine-deficient group.

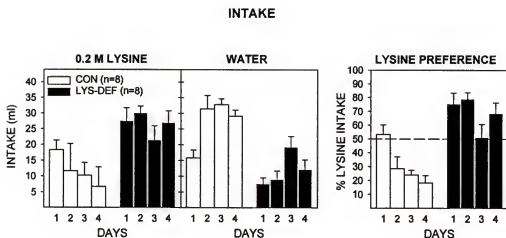


Figure 4-9. Experiment 2B: Two-Bottle Intake and Preference

Mean (+ SE) 23-h intake in milliliters of 0.2 M lysine (left panel) and water (middle panel) and the mean percent of total intake that was lysine (+ SE; right panel) all plotted by the four days of testing for control (CON; open bars) and lysine-deficient (LYS-DEF; closed bars) rats. These data were collected after intake tests with threonine and water.

resulting in a significantly greater preference for LYS ($F(1,14)=34.72$, $P<0.001$).

Consistent with the results from *Experiment 1A*, this group of LYS-DEF rats also took significantly more LYS bouts ($F(1,13)=10.11$, $P<0.01$) and fewer water bouts ($F(1,13)=17.35$, as LYS ($F(1,13)=35.42$, $P<0.01$)). The results for bout size were also congruous with those above; there were no differences between dietary groups for LYS ($F(1,10)=0.40$, $P=0.54$) or water ($F(1,8)=0.11$, $P=0.75$). Although the animals in this experiment were given experience with THR before they were ever presented with LYS solution, their intake and meal pattern results replicate those of *Experiment 2A*.

Experiment 2C: Lysine versus Threonine

When LYS and THR were presented together, LYS-DEF rats showed a pattern of behavior very similar to those described above for *Experiment 2A* (LYS versus water). In response to LYS deficiency, rats increased intake of LYS even in this two-amino acid choice situation (Figure 4-10). LYS-DEF rats drank significantly more LYS than CONS ($F(1,13)=8.35$ $p<0.01$) and less THR ($F(1,14)=8.86$, $p<0.01$) resulting in a significantly greater LYS preference ($F(1,13)=8.21$, $p<0.01$). Furthermore, there was a significant main effect of day for

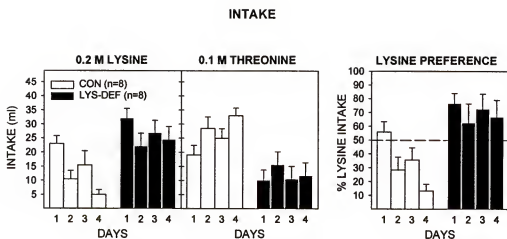


Figure 4-10. Experiment 2C: Two-Bottle Intake and Preference

Mean (+ SE) 23-h intake in milliliters of 0.2 M lysine (left panel) and 0.1 M threonine (middle panel) and the mean percent of total intake that was lysine (+ SE; right panel) all plotted for the four days of testing for control (CON; open bars) and lysine-deficient (LYS-DEF; closed bars) rats.

LYS intake ($F(3,39)=6.61$, $p<0.01$), THR intake ($F(3,42)=4.17$, $p<0.01$) and LYS preference ($F(3,39)=6.06$, $p<0.01$), but no interactions. Post hoc tests (collapsed over groups) revealed that both LYS intake and LYS preference were decreased on Days 2 and 4 relative to Day 1 (all $P<0.05$).

Bout number accounted for the increased intake (Figure 4-11); LYS-DEF rats took significantly more bouts of LYS ($F(1,12)=14.45$, $p<0.01$) and fewer to THR ($F(1,12)=19.58$, $p<0.01$) relative to CONs, resulting in a significantly greater percentage of LYS bouts ($F(1,12)=45.62$, $p<0.01$). There was a significant group by day interaction for both LYS bout number ($F(3,36)=4.99$, $p<0.01$) and the percentage of total bouts that were LYS ($F(3,36)=3.10$, $p<0.01$). Bonferroni-adjusted paired t-tests revealed that LYS-DEF rats only took significantly more bouts of LYS on Days 2 and 4, but LYS-DEF took a greater percentage of LYS bouts relative to CONs on all 4 days (all $P<0.05$).

Figure 4-12 reveals that bout size did not differ between the two dietary groups for LYS or THR. Ingestion rate (Figure 4-13) also did not differ between the groups for either stimuli. Collectively, these findings demonstrate that the meal pattern leading to enhanced LYS intake is altered in the same way (bout number is increased)

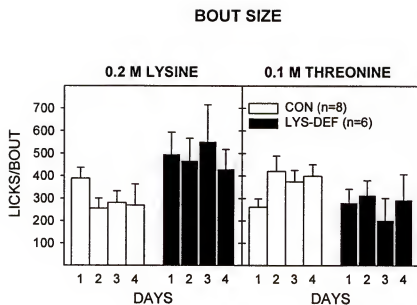


Figure 4-12. Experiment 2C: Bout size

Mean (+ SE) bout size in terms of licks per bout for 0.2 M lysine (left panel) and 0.1 M threonine (right panel) plotted over the four days of testing for control (CON; open bars) and lysine-deficient (LYS-DEF; closed bars) rats.

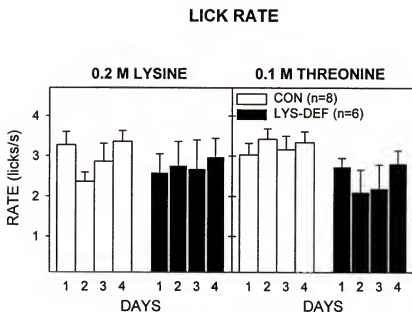


Figure 4-13. Experiment 2C: Lick rate

Mean (+ SE) lick rate represented as licks per second for 0.2 M lysine (left panel) and 0.1 M threonine (right panel) plotted by the four days of testing for control (CON; open bars) and lysine-deficient (LYS-DEF; closed bars) rats.

whether rats are presented with LYS and water or LYS and THR.

Figure 4-14 shows the number of cumulative licks that rats took during Day 1 of LYS and THR presentation. The LYS-DEF group took significantly more licks to LYS relative to CONs by 90 min after sampling the LYS (all $P < 0.05$); the groups differed for all later intervals, except minute 285 and 300. There was considerable overlap in THR cumulative licking; the CONs took significantly more cumulative licks to THR beginning at minute 855 (14 h, 15 min) and each interval thereafter. Interestingly, the addition of another chemical cue (i.e., THR) to the choice delayed the expression of the LYS appetite.

Discussion

Animals need to select balanced diets in their natural environments for optimal growth and reproduction. On a macrostructural level, these and previous experiments demonstrated that rats can successfully obtain needed nutrients, at least under the given experimental conditions. Specifically, rats fed an imbalanced diet consumed adequate quantities of LYS solution over days such that rate of growth became equal to that of CON rats fed nutritionally

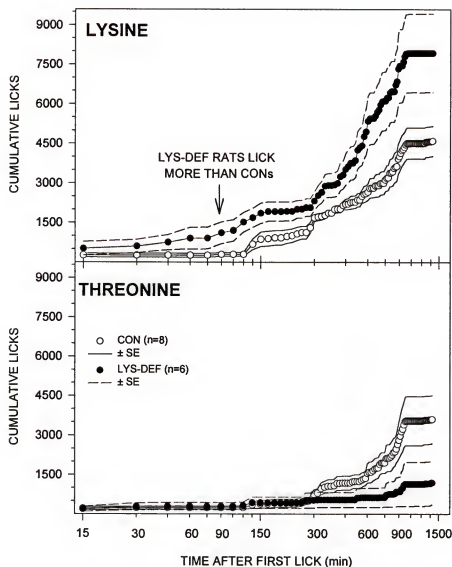


Figure 4-14. Experiment 2C: Cumulative licks on Day 1 of lysine presentation

Mean number of cumulative licks for 0.2 M lysine (top panel) and 0.1 M threonine (bottom panel) plotted in 15 minute intervals during the first 23-h amino acid presentation period. Time is plotted on a logarithmic scale to more clearly resolve the onset of the appetite. Controls (CON; open circles) take fewer cumulative licks to lysine and more to threonine relative to lysine-deficient (LYS-DEF; closed circles) rats. Solid lines represent SEs for controls. Dashed lines represent SEs for the lysine-deficient group.

complete amino acid diets. In the present experiments licking behavior was surveyed to better describe the behavioral basis of this enhanced LYS intake.

The analysis of the time course revealed that the enhanced intake of LYS in LYS-DEF rats occurred as early as 30 min after sampling, when LYS was pitted against water, and about 90 min after sampling, when LYS was pitted against another EAA, threonine. While this is a fairly rapid onset of LYS appetite, it is possible that within this time frame rats are able to form an association between the taste of LYS and its repleting postingestive consequences. LYS appetite, therefore differs substantially from sodium appetite since increases in sodium ingestion appear within seconds (Nachman, 1962; Handal, 1965), presumably not enough time for taste-visceral associations to form.

Interestingly, the presence of a second amino acid did not eliminate the development of a LYS preference. Its expression, however, was somewhat delayed. When LYS-DEF rats were given a choice between LYS and THR solutions, enhanced LYS intake appeared 90 min after rats had sampled the stimulus. This delay in preference can likely be attributed to the complexity of the discrimination. In *Experiment 2A* rats merely had to choose between a solution of water or LYS (a familiar stimulus and a "novel"

stimulus), whereas in *Experiment 2C* rats were required to choose between a solution of LYS or THR (two "novel" stimuli).

These results on the latency of LYS-DEF rats to ingest LYS parallel findings by other investigators in terms of detection of and behavioral reaction to dietary manipulations. When rats are fed amino acid imbalanced diets, diets that are disproportionately low in a single EAA relative to the other amino acids, they consistently show an anorectic response (e.g., Elvehjem & Krehl, 1955; Harper, 1958). In the present experiments, increases in LYS intake were taken as an index of the adaptive capabilities of the animals to respond to deficiency. An alternate strategy that has been widely used in the study of amino acid balance is to characterize the anorexia that rats exhibit in response to being fed an EAA-deficient (or imbalanced) diet. Interestingly, the emergence of the anorectic response occurs within time frames very similar to what has been shown here for enhanced solution intake. Leung and Rogers (1986) gave rats a choice of sufficient and THR-deficient diets. Between 2-3 h after diet presentation, rats expressed a preference for the balanced diet. In a similar experiment, Gietzen, Leung, Castonguay, Hartman, and Rogers, (1986) demonstrated decreased consumption of a THR-

deficient diet within 30-90 min after diet presentation. This more rapid behavioral response can be attributed to differences in experimental procedures; Gietzen, Leung, Castonguay, Hartman, and Rogers, (1986) presented only one diet to 18-h food deprived rats. The slightly different time courses between our experiment and those of others may be attributed to the differences in metabolic action among individual amino acids and/or modes of presentation (solid versus liquid). Collectively, these data support the notion that a fairly rapid mechanism for detection exists such that behavioral responding can occur to circumvent the negative consequences of prolonged amino acid deficiency.

As mentioned above, the ease with which LYS-DEF rats expressed a LYS preference when LYS was pitted against water (*Experiment 2A*) could be related to the fact that they were simply selecting the "novel" solution. Rodgers and Rozin (1966) have shown that rats made deficient in the vitamin thiamine preferred a novel diet to the familiar diet that was used to induce deficiency. They concluded that deficient rats may choose novel diets as a strategy to overcome nutritional deficiency. This raises the question of whether EAA-deficiency produces a chemospecific appetite or a general increase in consumption of any novel available substance. It is possible that the inception of a learned

preference for LYS is merely a preference for anything novel. Only after the association of LYS taste and repleting consequences does a lasting LYS preference develop. This possibility was investigated in *Experiment 2B* by a close examination of responsiveness to THR, a novel stimulus that does not lead to recovery in the LYS-DEF rat. If EAA deficiency merely stimulates an appetite for "novelty" then one would have expected LYS-DEF rats to (at least) initially show an increase in responsiveness to any chemical and only continue to ingest those that were associated with positive postingestive benefits. Although only ingestion in 15 min intervals was analyzed, there was no evidence of increased consumption of THR, the nonlimiting amino acid, at any time during testing. While the enhanced intake of LYS is presumably learned, these results demonstrate some degree of chemospecificity in behavioral responsiveness even initially.

Another important and consistent finding in the present series of experiments was that enhanced intake of LYS in LYS-DEF rats can be accounted for by an increase in bout number and not an increase in bout size. This may mean that LYS deficiency does not serve to elevate the "palatability" of LYS. The definition and utility of the term palatability has been the center of some debate (e.g., Ramirez, 1990;

Kissileff, 1990; Rogers, 1990). For our purposes palatability can be considered a hypothetical construct that cannot be directly observed but can be inferred from or operationally defined as certain behaviors including those described by meal pattern parameters (i.e., bout size, bout number) and within-bout ingestion rate. By way of comparison, it is useful to briefly review meal pattern analysis findings by other investigators that have used stimuli considered to be "palatable." Spector and Smith (1984) have shown that as the concentration of sucrose was increased (up to particular concentrations), nondeprived rats responded by increasing both bout size and bout number. Smith, Wilson, Krimm and Merryday (1987) showed that when rats were given access to 32% sucrose it was ingested in prolonged bouts relative to consumption of 0.2% sodium saccharin. It is thought that these alterations in meal pattern reflect increases in palatability. In contrast, the LYS-DEF rat consuming LYS increased only bout number and not bout size. It is tempting to speculate that under conditions of deficiency the hedonic value of LYS would increase; bout size and bout number would be elevated relative to water intake (in LYS-DEF rats) and relative to LYS intake in nondepleted control rats. But, this was not the case. Whether LYS-DEF rats were presented with a choice

between LYS and water, or between LYS and THR they consistently increased LYS intake as a function of bout number, not size.

That LYS deficiency does not appear to enhance the palatability of LYS is further supported by the analysis of lick rate. Lick rate has been used as an index of palatability (Davis, 1989; Davis & Smith, 1988). For example, when rats consumed sucrose (the archetype of palatability), within bout drinking rate increased with concentration (Spector and Smith, 1984). However, the LYS-DEF rat showed no differences in lick rate to LYS relative to water or nondeficient controls.

Collectively, the pattern analysis of ingestive behavior in LYS-DEF rats revealed the following: (1) LYS deficiency produces a specific appetite that is expressed relatively quickly but not immediately. In two bottle tests, the appetite emerged as early as 30 min following sampling when LYS was presented with water, and as late as 90 min when LYS was tested with THR. (2) LYS deficiency does not seem to enhance the palatability of the limiting amino acid as judged by behaviors thought to reflect palatability such as lick rate and bout size. Instead, it appears that LYS-DEF rats relieve the deficiency by increasing appetitive responding. Specifically, these

animals increased the total number of bouts of a LYS solution. These findings highlight the advantages of analyzing ingestion as a moment-by-moment behavioral event, instead of merely examining total output (e.g., intake).

CHAPTER 5 EFFECT OF NUTRIENT DEFICIENCY ON LEVER PRESSING

Introduction

The results from the gustometer tests (after rats had experience with repletion) were somewhat surprising. Following experience with the limiting amino acid on the home cage for a period of days, deficient rats were expected to express a preference (i.e., elevated licking to the deficient EAA relative to CONs and to the other stimuli in the array) in the short-term gustometer test. LYS-DEF rats did not show differences in licking to LYS during the second gustometer test (after six days of experience with the limiting amino acid and water on the home cage, when rats were thought to be EAA-replete) or during the third gustometer test, following 10 days of LYS-DEF diet, a period of time sufficient to induce depletion as indicated by body weight. While it is unlikely that the appetite for the limiting amino acid is innate, several questions remain unanswered from the results generated in *Experiments 1A* and *1B* with regard to LYS appetite. First, will a LYS-DEF rat

perform a learned response (e.g., lever-pressing) to obtain LYS? Additionally, how might prior experience with LYS and its repleting postingestive consequences influence the quantitative degree of the response?

In a series of classic experiments, Quartermain, Miller, and Wolf (1967) and Kriekhaus and Wolf (1968) examined the reinforcement efficacy of sodium in sodium-deficient rats. Water-deprived rats were trained to press a lever to receive small volumes of a liquid reinforcer, either water or salt. When the subjects were later sodium-depleted, they showed very robust lever-pressing responses either under extinction conditions (when they had been trained to press for a sodium salt) or when they received NaCl reinforcement at the time of testing. Those rats that were rewarded with water or nonsodium salts, or were not deficient during testing, displayed very little, if any, behavior. These experiments support the notion that sodium-depleted rats will perform an operant task to obtain a specific needed commodity. Moreover, physiological state affects the value of the given taste stimulus. The number of lever presses for the same stimulus (0.33 M NaCl) varied systematically as a function of degree of sodium-deficiency. Quartermain, Miller, and Wolf (1967) induced acute sodium deficiency with subcutaneous injections of formalin. As

formalin dose increased, so did rate of pressing. Certainly then, the value of a reward, at least as measured by lever pressing, is both a function of the reinforcer and the internal state of the organism.

Wolf and colleagues (1967, 1968) employed a variable interval operant schedule to examine interactions between physiological state and reinforcer. A progressive ratio (PR) schedule of reinforcement (Hodos, 1961) was adopted in these projects for both theoretical and practical reasons. With a PR schedule, the number of lever presses required to obtain a reward increases on each successive trial over the course of the testing session. A theoretical advantage of this type of schedule is that it provides a single measure of *reward strength*. This measure can be referred to as the "break point" and is operationally defined as the maximum number of responses an animal will make to obtain the reward. In simple terms, the PR schedule affords us the ability to determine how hard a rat will "work" to obtain a given reward in the context of a particular physiological state.

Another desirable feature of the PR schedule, relative to other operant schedules, is that depending on the step size only minimal amounts of the reinforcer are actually given to the animal. Motivated responding can be maintained

and assessed in depleted animals without rapid changes in physiological status. This is important since physiological state is dynamic and can be affected by the reinforcer itself. In the case of LYS deficiency, giving the rat significant quantities of LYS reward could potentially decrease deprivation level over the course of a single test session. This is circumvented or at least minimized with the PR schedule because the rat is only provided with small volumes of the stimulus per reward. More importantly, the reinforcement contingencies (number of lever presses) become stricter as testing progresses so overall the rat only receives a very small total stimulus volume.

Progressive ratio schedules have been used successfully to show changes in motivational status as a function of sweetened condensed milk concentration (Hodos, 1961), food deprivation state (Hodos, 1961), and treatment with the opioid antagonist, naloxone (Cleary, Weldon, O'Hare, Billington, & Levine, 1996). The present experiment was designed to begin to probe the effects of deficiency and experience on the reinforcing properties of the taste of LYS. Herein, experience consisted of LYS-DEF rats drinking LYS solution over 23-h periods to recover from their deficiency. Theoretically, then, the taste of LYS solution was associated with recuperation. Deficiency was varied to

demonstrate the role of nutritional status. In other words, some rats were LYS-DEF and others were fully recovered from a previous deficiency. Responses to sodium in sodium-deficient rats were also examined in order to establish the effectiveness of the PR procedure.

Experiment 3A: Sodium Deficiency and Lever Pressing

The nature of sodium appetite is understood in much greater detail compared to what is known about EAA appetite. As described in the general introduction, for example, rats depleted of sodium show a very robust, specific, and immediate (i.e., innate) appetite for sodium salts. The operant conditioning experiments by Wolf and his collaborators (1967, 1968) exemplify some of these aspects and reveal, particularly, that lever pressing is stimulated. Therefore, this model of deficiency was chosen to demonstrate the effectiveness of the PR lever pressing paradigm. In the case of negative or ambiguous findings in LYS-DEF rats, it would be necessary to illustrate the sensitivity of the PR paradigm to physiological state in a system where behavioral changes have previously been shown.

Method

Subjects

Fourteen male Sprague-Dawley rats (394 g $\pm$ 35.6 g) were used as subjects. Rats had ad libitum access to pelleted Purina Chow (5001) and distilled water except where otherwise noted.

Lever training and water deprivation testing

Rats were water-deprived for ~23 h and trained to press either one of two levers to receive 30 licks (~5 μ l/lick; a total of 0.15 ml per trial) of 0.15 M NaCl. Rats were trained to press the levers through the reinforcement of successive approximations to the target response. During this six-day training phase, a fixed ratio 1 (FR1) schedule of reinforcement was implemented. Thus, each time the lever was pressed the drinking spout was rotated into place and the reinforcer was available to the animal (i.e., 30 licks of 0.15 M NaCl). All rats learned to press the levers by the third day of training. Following FR1 training, rats were reinforced on a progressive ratio 1 (PR1) schedule for four days. In other words, the number of presses required for reinforcement increased by one relative to the prior requirement. The length of the session was determined by the rat; a termination criterion was set at 180 s such that when there were no lever-presses for 3 min the session was

terminated. On the whole, when rats were switched to the PR schedule, they did not receive a sufficient amount of fluid during the testing sessions to maintain stable body weight. Therefore, all rats were given 10 ml of supplemental water on *Days 8* and *9*.

The last three days of PR1 measurement served as the water-deprived baseline test (initial testing, before furosemide treatment). Water bottles were returned for two days and rats were tested for the next three days under nondeprived conditions. This testing phase served as the non-water-deprived testing condition. At the end of the experiment, after the furosemide treatments and testing, rats were tested one final time under water-deprivation.

Sodium depletion

The day before sodium depletion treatment, all rats were given sodium-deficient chow (Teklad 170905) in addition to their regular chow. This was done so that food-related neophobia would be minimized the following day during the sodium depletion procedure. Subjects were divided into two groups that were balanced as closely as possible for body weight and the number of ratios completed during baseline measurements. The rats in the sodium-deficient group were injected (i.p.) with 30 mg/kg body weight of the diuretic/natriuretic furosemide (Lasix). The drug was

administered in two equal doses, 2 h apart, with the first dose given 24 h prior to behavioral testing. At the time of the first injection, rats were moved to clean metabolism cages and allowed access to sodium-deficient chow and distilled water. The CON rats were treated in exactly the same manner but were injected (i.p.) with 0.15 M (isotonic) saline (0.3 ml/kg body weight). This sodium depletion procedure was implemented on two occasions. Following the first furosemide treatment, rats were tested for two days after depletion. Urine was collected for these two, 24 h periods. The concentration of sodium and potassium excreted was measured with a flame photometer (Ciba Corning 480) and total amounts of these solutes lost during the 24-h periods were computed. Rats were allowed to recover with ad libitum access to regular chow and distilled water for two days and treated with furosemide again to induce deficiency for a third testing session. The procedures for the second furosemide treatment were identical to those presented above. As before, urine was collected in order to compute the amounts of sodium and potassium excreted.

Testing after furosemide treatment

During testing, the number of PRs a rat completed to receive NaCl reinforcement was measured (i.e., PR break point). All subjects (non-water-deprived) were tested on

the PR1 schedule for two days after the first furosemide treatment and for one day after the second furosemide treatment. Rats were allowed to recover for one day, then water bottles were removed, and rats were tested a fourth and final time under water deprivation conditions. Table 5-1 provides an outline of the procedures of *Experiment 3A*.

Data analysis

Progressive ratio break points (i.e, the number of lever presses before the final reinforcement in a session) were statistically analyzed. Baseline testing (PR water-deprived and PR nondeprived) was conducted over three days for each physiological state. Two-way ANOVAs (group by day) were calculated for these two testing phases. Furthermore, the three days were averaged to provide one value for each condition (i.e, water-deprived and nondeprived). One-way ANOVAs were used to compare these average PR break point values between groups for each test condition, including the final water deprivation test. Two-way ANOVAs were used to determine if PR break point values varied for a given physiological state measured during different sessions (e.g., water-deprivation measured before and after the sodium depletion treatments). In the case of hydration state, there were three repeated measures so Bonferroni-

adjusted t-tests were done to compare response differences among sessions.

The total amount of sodium lost during the 24-h period immediately following the sodium-depletion procedure was computed as a function of the sodium concentration and the volume of urine excreted. Sodium lost was calculated for both furosemide-treated (i.e., sodium-deficient) rats and saline injected CONs after both depletion treatments. Rats in the sodium-deficient group that were injected with furosemide but did not lose sodium above the 95% confidence interval values of the CON group were excluded from the respective PR analysis. Two rats were excluded from the analysis of data collected after the first furosemide treatment and 1 rat was excluded from data pertaining to the second treatment.

Table 5-1. Procedure for Experiment 3A.

DAYS	PHASE	MANIPULATION
6	TRAIN PR1 WAT-DEP	rats were shaped to press either of two levers to receive 30 licks of 0.15 M NaCl while water-deprived
1	TRAIN PR1 WAT-DEP	rats were required to press a lever on a PR1 schedule to receive 30 licks of 0.15 M NaCl while water-deprived
3	BASELINE PR1 WAT-DEP	the number of responses made on a PR1 schedule of reinforcement, for 0.15 M NaCl, were measured while rats were water-deprived
3	BASELINE PR1 NO DEP	the number of responses made on a PR1 schedule of reinforcement, for 0.15 M NaCl, were measured while rats were not water-deprived
1	sodium-DEF chow habituation	rats were given sodium-deficient chow in addition to their Purina (5001) chow so as to rule out neophobia during the sodium-depletion procedure
1	sodium depletion treatment 1	rats were injected (i.p.) with either furosemide (SOD-DEF) or saline (CON) and moved to clean cages with ad lib access to water and sodium-DEF chow
2	PR1 TEST 1 and 2	the number of responses made on a PR1 schedule of reinforcement, for 0.15 M NaCl, were measured while rats were not water-deprived
1	sodium depletion treatment 2	rats were injected (i.p.) and moved to clean cages with ad lib access to distilled water and sodium-DEF diet.
1	PR1 TEST 3	the number of responses made on a PR1 schedule of reinforcement, for 0.15 M NaCl, was measured while rats were not water-deprived
1	PR1 TEST WAT-DEP	the number of responses made on a PR1 schedule of reinforcement, for 0.15 M NaCl, was measured while rats were water-deprived

Results

Two-way (group by day) ANOVAs were conducted to determine if break points varied as a function of days during non-water-deprived and water-deprived testing. There were no main effects of day or group and no interactions. Because PR break points did not vary as a function of days, for each animal the three-day average for each condition was calculated and these single scores represented the condition.

Rats that failed to be completely affected by furosemide were excluded from the data analysis. There were two rats excluded from the analyses of PR Test 1 & 2 and one rat excluded from the PR Test 3 data. All three rats were eliminated from the analysis of the final water deprivation test.

Figure 5-1 shows the PR break points when rats were tested under nondeprived and water-deprived conditions (the initial test and the final test). Individual one-way ANOVAs revealed that the groups did not differ during any of these tests; there were no group differences when rats were non-water-deprived or when water-deprived during either baseline testing or the final water-deprivation test. A two-way (group by session) ANOVA was used to examine changes in break point as a function of these three test sessions.

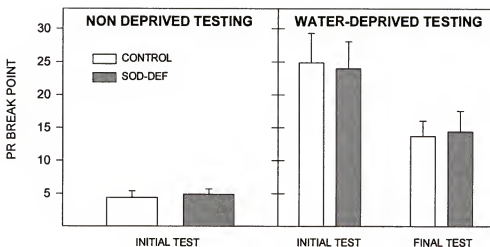


Figure 5-1. Experiment 3A: Effects of hydration state on progressive ratio break point for sodium chloride

Mean (+ SE) progressive ratio break point for 0.15 M NaCl when rats were non-water-deprived (left panel) and water-deprived during the initial and final tests (right panel). Groups (CON [open bars] and SOD-DEF [gray bars]) did not differ in response to either hydration state, but PR break point did vary as a function of test session.

As expected there was no effect of group or an interaction, but there was a significant main effect of session ($F(2,18)=27.33$, $P<0.001$). Bonferroni-adjusted t-tests (collapsed over groups), comparing the three test sessions, indicated that in both water-deprivation tests, rats had significantly higher break points relative to testing when rats were not water-deprived (all $P<0.05$). Furthermore, PR break point was decreased in the final water deprivation test relative to the initial water deprivation test ($P<0.05$). These results indicate that PR break point is sensitive to changes in hydration level.

Figure 5-2 represents the data from testing when rats were tested after furosemide treatment on two occasions. On all three test days the sodium-deficient groups had significantly higher break points relative to CONs (Day 1: $F(1,10)=6.25$, $P<0.05$; Day 2: $F(1,10)=21.09$, $P<0.001$; Day 3: $F(1,11)=8.55$, $P<0.01$). The three different sessions were compared in a two-way repeated measures ANOVA. Consistent with the individual one-way ANOVA results there was a significant main effect of group ($F(1,9)=6.34$, $P<0.05$). There was no difference, however, among testing sessions nor was there a significant interaction. These results signify that PR break point values reliably reflect sodium status.

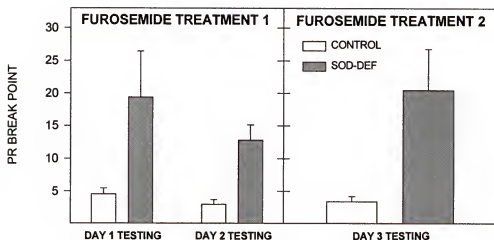


Figure 5-2. Experiment 3A: Effects of acute sodium depletion by furosemide treatment on progressive ratio break point for sodium chloride

Mean (+ SE) progressive ratio break point for 0.15 M NaCl when rats were made sodium-deficient with furosemide (SOD-DEF; gray bars) or injected with isotonic saline (CON; open bars). Rats were tested twice following the first furosemide treatment (left panel) and one time following the second treatment (right panel).

Experiment 3B: Lysine Deficiency and Lever Pressing

The results from *Experiment 3A* demonstrated that changes in physiological state can be detected by measuring PR break point for NaCl. Not only did rats have higher break points when they were sodium-deficient relative to when they were nondeficient, hydration state influenced level of responding for NaCl. Rats completed significantly fewer ratios when they were nondeprived compared to when they were water-deprived. Because the PR break point was successfully utilized to discern differences in physiological state with NaCl, this technique was employed with confidence in the following experiment using LYS. If LYS deficiency and/or experience with LYS does alter level of responding to LYS, this method should be able to elucidate such changes.

Method

Subjects

Twenty-four male Sprague-Dawley rats (269 g $\pm$ 8.6 g) were used as subjects. The rats had ad libitum access to pelleted Purina Chow (5001) during the first week of lever training and powered Purina Chow (5001) during the second week of the experiment.

Lever training and water deprivation testing

Lever training and baseline testing were conducted exactly as described above with the following exceptions:

- (1) Rats were reinforced with 0.2 M LYS instead of NaCl.
- (2) Three rats did not learn to press the levers by the fourth day of training and therefore were removed from the experiment (n=21).
- (3) On Day 4, 10 of the 21 rats were given 10 ml of supplemental water because their body weight dropped below 85% of their free feeding body weight. All subjects were given a 10 ml water supplement on Days 7, 8, and 9 because, in general, rats were unable to maintain body weight since fluid access was restricted to the testing sessions only.

Diet-induced lysine deficiency

Upon completion of lever training and testing during water-deprivation and non-water-deprivation, all rats were fed the basal diet for seven days and then the LYS-DEF diet for 10 days.

Repletion experience

Rats were allowed to recover from LYS deficiency either by access to the CON diet (see Table 2-1) or by access to 0.2 M LYS solution on the home cage for six days. Rats that were repleted by the CON diet represented the no experience (WAT-EXP) group because they received only water on the home

cage. Rats that were presented with LYS solution represented the LYS experience (LYS-EXP) group. The LYS-EXP group was presented with distilled water and 0.2 M LYS in graduated cylinders. The purpose of this manipulation was to provide these animals with the opportunity to associate the taste of LYS with its repleting postingestive consequences. Solutions were made and bottle positions were switched daily. The WAT-EXP group was simply presented with two graduated cylinders containing distilled water. Intake was measured each day.

Nutritional state for testing

Following the 6-day experience phase, half of the rats in each of these groups were then depleted of LYS again by 10 days access to the LYS-DEF diet alone. The other rats were fed the CON diet. This refeeding phase was done to manipulate nutritional state for testing (half were LYS-DEF and half were CON). The final groups and subject numbers are described in Table 5-3. Animals were divided into treatment groups closely balanced for body weight and PR break points obtained during the baseline measurements.

Testing

All subjects (non-water-deprived) were tested in the gustometers after the repletion experience phase and the nutritional state for testing phase (described above). The

number of PRs completed to receive LYS reinforcement was measured (i.e., PR break point) during three sessions, one per day for three days. During two additional test sessions (one per day for two days) the number of presses during a FR1 schedule was measured.

Data analysis

Progressive ratio break points (i.e, the number of presses in the final ratio during a given session) were statistically analyzed. Baseline testing (water-deprived and nondeprived) was conducted over three days for each physiological state. Two-way ANOVAs (group by day) were calculated for these two testing phases. The data were also averaged to provide one value for each condition (i.e, water-deprived and nondeprived). In addition, a two-way ANOVA was used to analyze the effects of hydration state.

Two-way ANOVAs were also used to compare break points between groups over PR test days and FR test days. Furthermore, the three-day average was calculated for PR testing and the two-average was calculated for FR testing. Individual differences among the groups were determined using Fisher's LSD post hoc tests. Fisher's LSD post hoc tests were used because although there were significant main effects of group using ANOVA, more conservative post hoc

tests did not reveal any differences among the individual groups.

Body weights were converted to percent of initial body weight by dividing each animal's body weight on a given day by that animal's body weight on the day before the basal diet was presented. These values were then analyzed using group by day ANOVAs for the different phases of the experiment. For significant main effects of day, paired t-tests were performed comparing days, collapsed over groups. When there were significant main effects of both group and day or group by day interactions, one-way ANOVAs with Tukey HSD post hoc comparisons were conducted for each day.

Table 5-3. Procedures for Experiment 3B.

DAYS	PHASE	LYS-EXP: LYS-DEF N=6	WAT-EXP: LYS-DEF n=5	LYS-EXP: CONTROL n=5	WAT-EXP: CONTROL n=5
6	TRAIN FR1 WAT-DEP	lever training 0.2 M LYS water-dep	lever training 0.2 M LYS water-dep	lever training 0.2 M LYS water-dep	lever training 0.2 M LYS water-dep
1	TRAIN PR1 WAT-DEP	PR1 training 0.2 M LYS water-dep	PR1 training 0.2 M LYS water-dep	PR1 training 0.2 M LYS water-dep	PR1 training 0.2 M LYS water-dep
3	BASELINE PR1 WAT-DEP	baseline PR1 0.2 M LYS water-dep	baseline PR1 0.2 M LYS water-dep	baseline PR1 0.2 M LYS water-dep	baseline PR1 0.2 M LYS water-dep
3	BASELINE PR1 NO DEP	baseline PR1 0.2 M LYS no water-dep	baseline PR1 0.2 M LYS no water-dep	baseline PR1 0.2 M LYS no water-dep	baseline PR1 0.2 M LYS no water-dep
7	BASAL diet feeding	basal diet water	basal diet water	basal diet water	basal diet water
10	LYS-DEF diet feeding	LYS-DEF diet water	LYS-DEF diet water	LYS-DEF diet water	LYS-DEF diet water
6	REPLETION	LYS-DEF diet 0.2 M & water	control diet water	LYS-DEF diet 0.2 M LYS & water	control diet water
10	PHYSIO STATE FOR TESTING	LYS-DEF diet water	LYS-DEF diet water	control diet water	control diet water
3	PR1 TEST	test (PR1) LYS reinforcer no water-dep	test (PR1) LYS reinforcer no water-dep	test (PR1) LYS reinforcer no water-dep	test (PR1) LYS reinforcer no water-dep
2	FR1 TEST	test (FR1) LYS reinforcer no water-dep	test (FR1) LYS reinforcer no water-dep	test (FR1) LYS reinforcer no water-dep	test (FR1) LYS reinforcer no water-dep

Results

Two-way (group by day) ANOVAs were conducted to determine if break points varied as a function of days during non-water-deprived and water-deprived testing. There were no main effects of day or group and no interactions. Figure 5-3 shows the three-day average PR break points during baseline testing when rats were water-deprived and nondeprived. Consistent with the two-way ANOVAs, individual one-way ANOVAs revealed that the groups did not differ when tested in either hydration state.

A two-way ANOVA was done to compare the two hydration levels (nondeprived and water-deprived). As is obvious in Figure 5-3, break points were significantly higher when rats were water-deprived relative to when they were not ($F(1,17)=190.0$, $P<0.001$). There was no main effect of group nor an interaction. As was observed when NaCl was the reinforcer, PR break point also reflected hydration state when rats were reinforced with LYS.

Figure 5-4 represents the PR break points when rats were tested after the experimental manipulations; panel 1 depicts the data for each of the three test days and panel 2 depicts the three-day mean values. A group by day ANOVA indicated no group by day interaction or main effect of day,

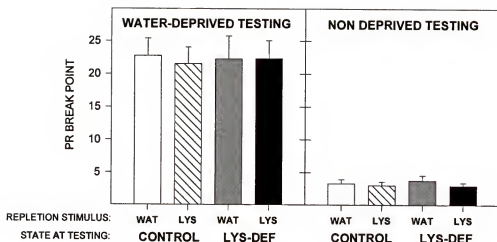


Figure 5-3. Experiment 3B: Effects of hydration state on progressive ratio break point for lysine

Mean (+ SE) progressive ratio break point for 0.2 M lysine when rats were water-deprived (left panel) and non-water-deprived (right panel). Although rats are divided into groups, the results depicted here were obtained prior to dietary and experience manipulations.

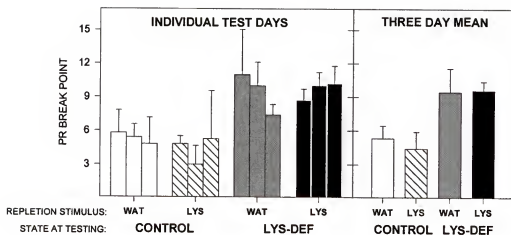


Figure 5-4. Experiment 3B: Effects of experience and physiological state on progressive ratio break point for lysine

Mean (+ SE) progressive ratio break point for 0.2 M lysine for rats that 1) were repleted through diet and not deficient during testing (WAT:CON; open bars), 2) were repleted with lysine solution and not deficient during testing (LYS:CON; right-hatched bars), 3) were repleted through diet and lysine-deficient during testing (WAT:LYS-DEF; grey bars), 4) were repleted with lysine solution and lysine-deficient during testing (LYS:LYS-DEF; closed bars). Progressive ratio breakpoints are plotted for each of the three test days (left panel) and as the average across the three test days (right panel).

but did reveal a significant effect of group ($F(3,16)=3.57$, $P<0.05$). Fisher's LSD tests, using the three-day mean PR break point values, indicated that both LYS-DEF groups had significantly higher PR break points relative to the LYS:CON group; only the LYS:LYS-DEF group had significantly higher break points than the WAT:CON group (all $P<0.05$). According to PR break point values, only physiological state (LYS-DEF versus CON) affected behavior. There were no differences between groups that were given exposure to LYS solution to cause repletion relative to those that were simply given water.

Figure 5-5 illustrates the effect of experience with LYS solution and physiological state at time of testing on the number of presses animals made when on the FR1 schedule of reinforcement (conducted the two days following PR testing). Panel 1 of Figure 5-5 shows data from each of the two test days and panel 2 shows the two-day average values. A group by day ANOVA revealed neither an effect of day nor a group by day interaction. Consistent with the PR testing, there was a significant main effect of group ($F(3,17)=13.08$, $P<0.001$) when rats were tested on a FR1 schedule of reinforcement. Fisher's LSD tests on the two-day average number of presses (Figure 5-5, panel 2) showed that both LYS-DEF groups had significantly higher PR break

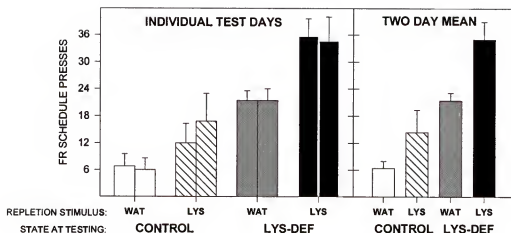


Figure 5-5. Experiment 3B: Effects of experience and physiological state on fixed ratio pressing for lysine

Mean (+ SE) number of presses made on a fixed ratio schedule of reinforcement for 0.2 M lysine for rats that 1) were repleted through diet and not deficient during testing (WAT:CON; open bars), 2) were repleted with lysine solution and not deficient during testing (LYS:CON; right-hatched bars), 3) were repleted through diet and lysine-deficient during testing (WAT:LYS-DEF; grey bars), 4) were repleted with lysine solution and lysine-deficient during testing (LYS:LYS-DEF; closed bars). Number of presses are plotted for each of the two test days (left panel) and as the average of the two test days (right panel).

points relative to the LYS:CON group (all $P < 0.05$). Also, the two LYS-DEF groups differed; the LYS:LYS-DEF group pressed more than the WAT:LYS-DEF group (all $P < 0.05$). These later results suggest that in addition to the effect of LYS deficiency, experience is playing a role in this lever pressing behavior.

The body weight data (represented in terms of percent of initial body weight) for the four groups are presented in Figure 5-6. Separate sets of analyses were conducted for each of the five phases defined on the figure.

A group by day ANOVA for the basal diet feeding phase revealed that all groups of rats gained weight over the seven days of measurement ($F(6,102)=130.0$, $P < 0.001$). Paired t-tests, collapsed over groups, indicated that body weight significantly increased each day (all $P < 0.05$) except on Day 1 to Day 2. During the 10 days when all rats were fed the LYS-DEF diet, body weight significantly dropped over days ($F(9,144)=30.92$, $P < 0.001$). Each day, the body weight was significantly lower relative to the day before (all $P < 0.05$) except for Days 6, 7, and 8. During the repletion phase of the experiment, mode of repletion (i.e., eating a complete diet or drinking LYS solution) had no influence on body weight and all groups gained weight over the six-day repletion period ($F(5,80)=113.0$, $P < 0.001$). Paired t-tests

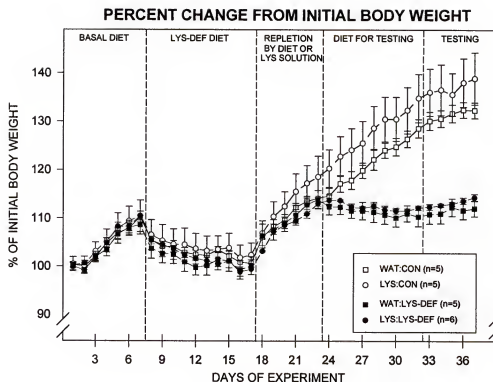


Figure 5-6. Experiment 3B: Body Weight

The mean ($\pm$ SE) percent of initial body weight plotted as a function of day during the experiment. The experiment has been divided into five phases: Phase 1, all groups were fed the basal diet; Phase 2, all groups were fed the lysine-deficient diet; Phase 3, two groups (LYS:CON and LYS:LYS-DEF) were repleted by access to 0.2 M lysine on the home cage for 6 days and the other two groups (WAT:CON and WAT:LYS-DEF) were given only water on the home cage but repleted with the lysine-sufficient CON diet; Phase 4, physiological state for testing was induced using either the lysine-sufficient control diet (LYS:CON and WAT:CON) or the lysine deficient diet (LYS:LYS-DEF and WAT:LYS-DEF); Phase 5, all rats were tested across 5 days.

showed that the percent of initial body weight increased from day to day (all $P < 0.05$). In the fourth phase, rats were given different diets (CON or LYS-DEF) based on their assigned physiological state during testing. A group by day ANOVA for the nine days of the "diet for testing" phase revealed significant main effects for group ($F(3,17) = 10.47$, $P < 0.001$) and day ($F(8,136) = 59.02$, $P < 0.001$) and a significant interaction ($F(24,136) = 35.61$, $P < 0.001$). One-way ANOVAs, conducted for each day, indicated significant group differences on Days 2 through 9 (see table 5-3 for F values). Tukey post hoc tests showed that the LYS:CON group was significantly larger than both LYS-DEF groups on Days 2 through 9 (all $P < 0.05$). Additionally, the WAT:CON differed from the WAT:LYS-DEF group on day 6 and each day thereafter, and the LYS:LYS-DEF on Days 7, 8, and 9 (all $P < 0.05$). Body weight of the two CON groups did not differ on any day during this phase of the experiment. For the final phase of the experiment, testing, there were significant main effects of group ($F(3,17) = 23.15$, $P < 0.001$) and day ($F(4,68) = 15.19$, $P < 0.001$). The group by day interaction only approached significance. One-way ANOVAs conducted at each day revealed that the body weight differed among groups on each test day (see table 5-3 for F values). Tukey post hoc comparisons indicated that the percent of initial body weight was always

greater in both CON groups relative to both LYS-DEF groups (all $P < 0.05$).

Table 5-3. One-way ANOVA results for body weight during phases four and five.

Day	pre-test feeding	testing
1	$F(3,17)=2.32$, ns	$F(3,17)=22.14$, $P < 0.001$
2	$F(3,17)=4.26$, $P < 0.05$	$F(3,17)=20.62$, $P < 0.001$
3	$F(3,17)=5.75$, $P < 0.01$	$F(3,17)=25.04$, $P < 0.001$
4	$F(3,17)=7.53$, $P < 0.01$	$F(3,17)=24.22$, $P < 0.001$
5	$F(3,17)=10.04$, $P < 0.001$	$F(3,17)=21.91$, $P < 0.001$
6	$F(3,17)=14.69$, $P < 0.001$	
7	$F(3,17)=15.96$, $P < 0.001$	
8	$F(3,17)=17.72$, $P < 0.001$	
9	$F(3,17)=19.73$, $P < 0.001$	

Discussion

The main purpose of the experiments presented herein was to examine the reinforcement efficacy of LYS in rats with different immediate nutrient needs (LYS-DEF versus LYS-replete) and with different associative histories with LYS solution and repletion. Because a testing paradigm that has rarely been used was adopted for this project, a related question was also addressed: what is the reward value of NaCl in sodium-deficient relative to CON rats?

The induction of sodium appetite through deficiency proved to be an effective model to demonstrate the sensitivity of PR break point to differences in physiological state. Consistent with Quartermain, Miller, and Wolf (1967) and Kriekhaus and Wolf (1968), the reinforcement efficacy of NaCl was magnified by sodium deficiency even when a PR1 schedule of reinforcement was used. Furthermore, this measure of *reward strength* appears to produce reliable outcomes. On three separate days rats were tested yielding consistent results; sodium-deficient rats had significantly higher PR break points than their nondepleted counterparts (see Figure 5-2). Progressive ratio break point was also affected as would be expected by water deprivation. Break point showed roughly a sixfold increase when rats were water-deprived relative to when they had ad libitum access to water prior to testing (see Figure 5-1).

Interestingly, data from the PR paradigm failed to support the hypothesis that responsiveness to NaCl is increased upon repeated furosemide treatments. Sakai, Fine, Frankmann, and Epstein (1987) found that having once been depleted of sodium, intake of NaCl in the rat was increased significantly when either tested when depleted or under need-free circumstances (Sakai, Frankmann, Fine, & Epstein,

1989). No differences in PR break point were found after a second furosemide treatment. The comparison of the results from this experiment with those of Sakai, Fine, Frankmann, and Epstein (1987) must be made cautiously because different methods of measurement were used. In the present experiment lever-pressing was measured using a PR1 schedule of reinforcement, whereas Sakai and colleagues (1987, 1989) measured long-term intake. Although there may be a physiological change in the brain as a result of repeated sodium depletion, break point may not serve as a sensitive enough behavioral correlate or perhaps more depletions are required before the threshold for change in responding on a PR1 schedule would be reached.

Although PR break point did not reflect changes in responsiveness with repeated furosemide administration, it is undoubtedly sensitive to overall changes in sodium status. Thus, the data from *Experiment 3A* have successfully served the purpose of ensuring that the PR procedure is sensitive to or can reflect changes in physiological state.

By comparing lever-pressing responsiveness between LYS-DEF rats that did and did not have experience with pairing LYS solution and recovery from deficiency (LYS:LYS-DEF and WAT:LYS-DEF), the effect of experience with LYS solution on behavioral responding in this paradigm was examined. In

other words, is the reward value of LYS heightened through experience? Is experience with repletion from a LYS-deficient state necessary for a LYS appetite to be expressed in the PR paradigm or is it simply the physiological state during testing that determines responsiveness?

In contrast to the lack of evidence of a conditioned response in the gustometer tests of *Experiment 1A*, it was predicted that when LYS-DEF rats are given the chance to associate LYS solution with repletion (LYS:LYS-DEF) in this experiment they would press significantly more relative to the deficient rats that had no prior experience with LYS solution during recovery from deficiency (WAT:LYS-DEF). Surprisingly though, there was no evidence of this effect when rats were tested using the PR1 schedule (see Figure 5-4). However, increased responding was observed in the LYS:LYS-DEF group relative to the WAT:LYS-DEF group when rats were later tested on a FR1 schedule (see Figure 5-5).

There are at least two possible explanations for these results. First, it is conceivable that rats were not trained enough on the PR1 schedule before testing was conducted and were not fully familiar with the reinforcement schedule. A second possibility (assuming less emphasis on the results from FR testing) is that LYS deficiency produces a general increase in overall responsiveness. In turn, both

LYS-DEF groups showed similar levels of responding and experience plays only a small role above and beyond this. Additional experiments could be conducted to examine this hypothesis. In other words, would both groups of LYS-DEF rats, those with experience and those without, show elevated PR break points to another seemingly irrelevant stimulus such as THR? This increase in appetitive behavior is consistent with other findings presented previously. For example, in *Experiments 1A and 1B*, both groups of EAA-deficient rats initiated significantly more trials during all gustometer tests relative to the nondepleted CONs. Furthermore, in *Experiment 2A and 2C* LYS-DEF rats increased intake of LYS by increasing bout number. The initiation of ingestion was more frequent compared to CONs. This response was somewhat specific, however, since the deficient rats only showed this response to LYS, and not WAT or THR.

A comparison between the two groups that had no prior exposure to LYS solution (WAT: LYS-DEF and WAT:CON) demonstrates the drive dependence of the unconditioned response. In other words, physiological state during testing, for the most part, determined the level of responding. Under both PR1 and FR1 schedules, LYS-DEF rats pressed the lever significantly more relative to nondeficient CONs.

In summary, these experiments: (1) demonstrate that a PR1 schedule can be successfully used to exhibit differential responding as a result of physiological state (hydration level and specific nutrient deficiency), (2) indicate that both LYS and NaCl can act at least as primary reinforcers in a lever-pressing paradigm under conditions of nutrient deficiency and water deficiency, and (3) reveal that the value of LYS and NaCl is primarily dependent on physiological state (for LYS, reinforcer value can be modified by experience at least as measured using a FR1 schedule of reinforcement). Future experiments should be conducted that address whether the increase in appetitive behavior occurs only in the context of the limiting nutrient or is general to any stimulus presented.

CHAPTER 6 GENERAL DISCUSSION

The work presented here could potentially serve as a framework for other investigations of the "body wisdom" concept in animal models. In accordance with Cannon's ideas (1932), the findings of this dissertation support the premise that animals are capable of ingesting appropriate foods based on nutritional requirement. Specifically, rats deprived of an EAA (either LYS or THR) engage in behavioral mechanisms to guide recovery. Additionally, these experiments highlight a number of important aspects of how this recovery is mediated in the LYS-DEF rat. It is hoped that the features described here can be applied to recovery from other specific micronutrient deficiencies.

At this point I would like to summarize and integrate the collective findings of this experimental examination of the LYS-DEF rat. Using one of the simplest measures applied in the study of feeding behavior, total (long-term) intake, these studies have shown that LYS-DEF rats drink significantly more LYS than nondeficient CONs such that recovery is incurred. Recovery is indicated by a gain in

body weight relative to periods of no growth or weight loss when rats only have access to the deficient diet. Even in this somewhat contrived experimental situation, where the essential nutrient is administered in solution form, deficient rats repeatedly demonstrated this response. Although the fact that rats drink enough LYS solution to support recovery (as indicated by body weight gain) when the nutrient is simply presented on the home cage in addition to water is compelling, that these rats are able choose LYS even when it is pitted against another chemical stimulus, THR, provides a convincing example of the adaptive nature of the appetite.

While this appetite is easily and consistently displayed in long-term intake tests, there is no evidence for the expression of the appetite in brief-access licking tests. The most obvious explanation for this is that appetite for LYS is not innate (like sodium appetite). In simple terms, the LYS-DEF rat does not "know" immediately (based on the sensory features of LYS) what stimulus to ingest in order to replete. This hypothesis is further supported by the time course leading to elevated LYS intake (measured in the 23-h intake tests). Sodium-deficient rats presented with sodium increase licking within seconds relative to controls (e.g., Handal, 1965); LYS-DEF rats

(when LYS is presented with water) do not show heightened responsiveness until 30 minutes after sampling. The discrimination is made more complex when LYS is presented simultaneously with THR. As a result, augmented licking takes, on average, 90 minutes to occur. This argument is best applied to results from tests that measure the onset of responding in naive animals that have never experienced LYS deficiency or LYS solution.

In subsequent experiments, an effort was made to examine what effect preexposure to the stimulus (in the context of recovery from depletion) would have on responding. For example, I predicted that rats would develop a conditioned response to LYS when tests were conducted following experience with LYS on the home cage. Specifically, it was thought that (1) LYS-DEF rats would differentially increase licking to LYS in the later gustometers tests and (2) have elevated PR break points relative to rats with no experience. During "experience," LYS-DEF rats drank relatively large quantities of LYS over 23-h periods and showed signs of recovery from the deficiency (i.e., increases in body weight). The premise was that drinking LYS while depleted would allow the taste of LYS to be paired with its repleting postingestive consequences and result in a conditioned response. However,

LYS-DEF rats did not increase licking to LYS in post-experience gustometer tests, nor did THR-DEF rats alter responding when recovery was induced by consumption of THR. Furthermore, PR break points obtained in the lever pressing experiment were not higher in rats that had experience with LYS relative to those animals who were repleted through dietary means and did not have access to LYS solution. A tenable hypothesis for the mechanism that supports recovery is that EAA deficiency stimulates a general activation in appetitive responding. In other words, deficient rats do not "learn" a *specific* response to a *specific* stimulus. This is unlikely, however, because the increase in appetitive behavior observed here is not completely general. Increases in appetitive behavior are not observed in the absence of the "correct" stimulus. For example, LYS-DEF rats do not take more licks or increase bout number to THR. Instead, it is apt that there is an interaction between heightened appetitive behavior and the presence of the necessary stimulus. In support of this, all cases where appetitive behaviors were increased, LYS was present (i.e., number of trials in the gustometer tests, number of bouts during 23-h intake, high PR break point).

It is noteworthy that the behavioral findings in the LYS-DEF rat conform to some extent to Berridge's (1996)

categorization of the rewarding aspects of food. Berridge has hypothesized that food reward contains two distinguishable psychological components: (1) "wanting"--the appetite/incentive motivation value of the stimulus and (2) "liking"--a reflection of the hedonic value of the stimulus. Moreover, he asserts that these functional components can be manipulated and measured independently. Typically, dependent measures such as total intake, preference, and operant responding are used to make inferences about the affective component of ingestive behavior. These measures, however, can represent both the hedonic (i.e., liking/palatability) and appetite/craving (i.e., wanting) aspects of reward. Specifically, the assumption that wanting = liking is wrong. Instead, Berridge believes that taste reactivity is a measurement most reflective of palatability (the liking component of food reward) and appetitive behaviors, that is, behaviors that require "capturing" the stimulus are more indicative of the wanting aspect of ingestion. While licking could be assigned to either category, once initiated it is probably better categorized as consummatory. Within this framework, the findings from this series of experiments suggests that while LYS-DEF rats want LYS they do not necessarily like it more.

In conclusion, I believe that investigating behavioral

recovery from specific EAA deficiency has important implications from a nutritional standpoint. To survive, an animal must eat a diet that includes enough total energy to maintain metabolic processes, some essential fatty acids, vitamins and minerals, and the basic building blocks of protein, EAAs. On the other hand, organisms do not specifically "require" carbohydrates and other nonessential fats because glucose and/or ketones can be generated by processes of catabolic breakdown and anabolic biotransformation from a variety of ingested resources. It is interesting then, that many investigators have undertaken the study of macronutrients besides protein (e.g., appetite for carbohydrate and fat) while fewer studies have focused on the critical macronutrient (e.g., protein or specific EAAs). For these reasons I chose a model involving EAAs and have characterized some of the behavioral mechanisms of recovery from deficiency. In the future it will be important to synthesize these behavioral findings with some of the anatomical and neurochemical mechanisms mediating EAA deficiency.

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BIOGRAPHICAL SKETCH

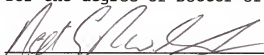
I was born on March 12, 1969 in Oakland, California. I attended public schools from kindergarten through twelfth grade. At 18 years, I moved south to attend college at San Diego State University where, in 4 years, I received a B.A. degree in psychology (1991). My interest in scientific research developed during the later years of my undergraduate education. As such, I entered the master's program at SDSU and worked in the laboratory of Dr. Claire Murphy. For my thesis, I used psychophysical techniques to examine gustatory and olfactory functioning across the life span. Upon completion of my M.A. in experimental psychology (1993), I began doctoral studies in psychobiology at the University of Florida under the guidance of Dr. Alan C. Spector. Since then, I have come to know the challenges of relating physiology to behavior. Upon completion of the Ph.D. degree, I will begin a postdoctoral position at the University of Pennsylvania and continue to integrate biology and behavior as applied to feeding.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.



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